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PROTEIN ALTERATION IN PORTAL CIRRHOSIS AS DETERMINED BY ELECTROPHORESIS

JOHN F. WHITMAN, M.D., H. R. ROSSMILLER, M.D., AND LENA A. LEWIS, PH.D.
CLEVELAND, OHIO

THE function of the liver in the processes of protein metabolism is well established and is becoming increasingly important in clinical medicine with the accumulation of additional information. Our observations include a study of the electrophoretically determined protein changes in portal cirrhosis, a statistical comparison of these with normal values, and a correlation of the abnormal levels with the concomitantly determined results of other liver function tests.

Methods of measuring serum proteins by precipitation or "salting out" have resulted in assumptions which must be revised to include the valuable data revealed by electrophoretic analysis. Chemically determined serum proteins differ from electrophoretic results in that somewhat higher average albumin and lower average globulin levels are obtained.[1] Total globulin levels may be within normal limits for plasma in which electrophoretic measurements show definitely altered values for individual globulin constituents. This situation is particularly true in early cirrhosis and in acute parenchymatous hepatic lesions. The alteration or inversion of the albumin-globulin ratio in liver disease has been considered significant universally and the presence of hypoalbuminemia has been stressed,[2] but further investigation of the alteration of other fractions and the significance of these changes seems warranted.

MATERIALS AND METHODS

Subjects.—Normal control values for the method were calculated on plasma from thirty-two individuals who were free of symptoms and abnormal physical findings and whose ages ranged from 20 to 48 years. The averages of these estimates, as listed in Table I, closely approximate those reported by Longsworth and co-workers,[3] Moore and Lynn as cited by Abramson and co-workers,[4] Gray and Barron,[1] Gutman,[5] and Luetscher.[6]

Thirty-nine patients with portal cirrhosis, ranging in age from 19 to 66 years, were observed. Diagnoses were verified in nineteen of these by autopsy, biopsy, peritoneoscopy or laparotomy, and a moderately extensive though not uniform liver function test schedule was accomplished in each case.

Method.[7]—Fasting blood samples, oxalated with 1.5 mg. potassium oxalate per milliliter of blood, were used. Plasma was removed after immediate high-speed centrifugation and total plasma nitrogen was determined by the Pregl[8] modification of the micro-Kjeldahl method. If the blood urea nitrogen was within normal limits, a nonprotein nitrogen correction factor of 25 mg. per 100 ml. was used; if the blood urea nitrogen was elevated, the

From the Department of Medicine and the Research Division of the Cleveland Clinic and the Frank E. Bunts Educational Institute.
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TABLE I. SUMMARY: PLASMA PROTEIN FRACTIONS IN NORMAL SUBJECTS

	TOTAL PROTEIN (GM.)*	ALBUMIN		α-GLOBULIN		β-GLOBULIN		γ-GLOBULIN		ø-GLOBULIN	
		GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†
Mean	6.55	4.12	62.8	0.47	7.07	0.88	13.5	0.76	11.5	0.33	5.1
Range	5.94-7.82	3.72-5.11	60.1-67.2	0.32-0.66	5.0-8.7	0.65-1.16	11.0-15.9	0.55-0.91	7.7-14.8	0.16-0.48	2.8-7.8
Standard deviation $\left(\sqrt{\frac{\Sigma x^2}{N}}\right)$	0.484	0.347	2.089	0.078	0.876	0.127	1.342	0.094	1.428	0.084	1.332
Standard error of mean $\left(\frac{S.D.}{\sqrt{N}}\right)$	0.0856	0.0614	0.3693	0.0138	0.1548	0.0224	0.2371	0.0165	0.2524	0.0147	0.2355

*Grams per 100 ml. plasma.
†Per cent of total plasma protein.

TABLE II. SUMMARY: PLASMA PROTEIN FRACTIONS IN PORTAL CIRRHOSIS

	TOTAL PROTEIN (GM.)*	ALBUMIN		α-GLOBULIN		β-GLOBULIN		γ-GLOBULIN		ø-GLOBULIN	
		GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†
Mean	6.87	3.01	42.5	0.53	7.7	1.23	17.9	1.44	21.1	0.70	10.2
Difference from normal mean	+0.32	−1.11	−20.3	+0.06	+0.63	+0.35	+4.4	+0.68	+9.6	+0.37	+5.1
Range	5.05-9.03	1.91-4.63	31.7-63.9	0.24-1.15	3.7-13.2	0.63-2.18	10.5-24.4	0.41-2.79	7.9-41.2	0.29-1.55	4.5-24.0
Standard deviation $\left(\sqrt{\frac{\Sigma x^2}{N}}\right)$	1.041	0.721	7.929	0.197	2.218	0.307	3.553	0.529	7.036	0.272	3.86
Standard error of mean $\left(\frac{S.D.}{\sqrt{N}}\right)$	0.1653	0.1154	1.2696	0.0315	0.3551	0.0491	0.5689	0.0846	1.1266	0.0447	0.634
Standard error of the difference of the means $\left(\sqrt{\sigma^2 M_1 + \sigma^2 M_2}\right)$	0.186	0.131	1.322	0.034	0.387	0.054	0.616	0.086	1.155	0.047	0.676
Difference/standard error of the difference $\left(\frac{D}{\sigma D}\right)$	1.72	8.5	15.35	1.75	1.63	6.48	7.14	7.89	8.32	7.87	7.54

*Grams per 100 ml. plasma.
†Per cent of total plasma protein.

nonprotein nitrogen was determined and the proper correction made. The value 6.25 was the protein factor used. Plasma was diluted with phosphate buffer of pH 7.8 and ionic strength 0.16 so that the resulting protein concentration was 1.4 Gm. per 100 milliliters. The diluted plasma was dialyzed for seventy-two hours at approximately 3° C. in a cellophane sack against the phosphate buffer solution. After dialysis, the diluted plasma was centrifuged at high speed. The Tiselius[9] electrophoresis technique as modified by Longsworth[10] was used.

RESULTS

Because of the extent of the record and the calculations involved, Table I is presented as a statistical summary of the electrophoretic analysis of the plasma from the thirty-two normal subjects. Means and range of values, standard deviations ($\sigma = \sqrt{\frac{\Sigma x^2}{N}}$), and standard errors of the means (S.E. $= \sigma/\sqrt{N}$) are listed. Table II includes the same observations for the group of thirty-

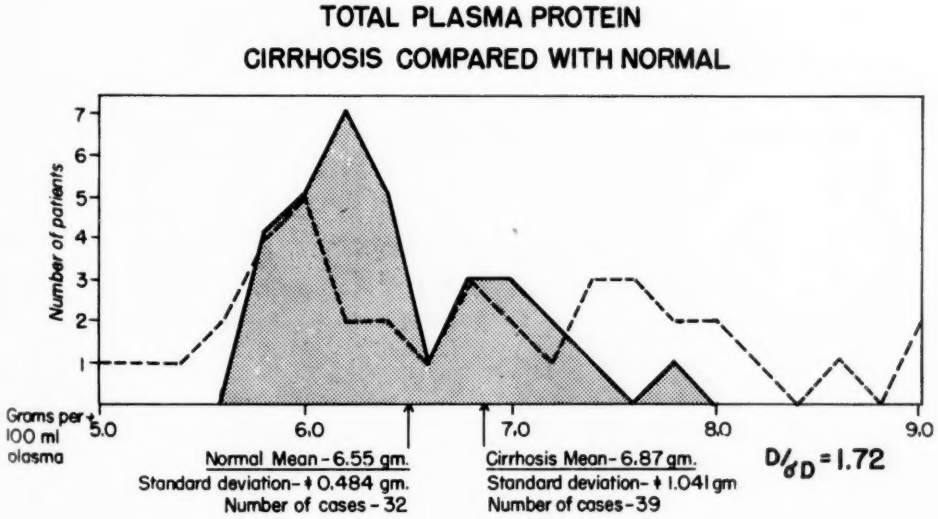

Fig. 1.

nine patients with portal cirrhosis and, further, the error of the difference between the means of the normal and cirrhosis groups ($\sigma_D = \sqrt{\sigma^2 M_1 + \sigma^2 M_2}$) and the estimation of the degree of statistical significance of these differences ($D/\sigma D$).

The results show the level of total plasma protein to be higher in patients with cirrhosis than in normal subjects but the difference is not statistically significant (Fig. 1). Plasma albumin levels were notably lower in the group with cirrhosis and the degree of statistical significance was great ($D/\sigma D =$ 8.5 in grams per 100 ml. and 15.35 in per cent of total protein as shown in Fig. 2). There was no significant difference in the α-globulin observations in the two groups. (Fig. 3.)

Differences in the levels of β- and γ-globulin were definitely statistically significant. There were striking increases, both absolute and relative, in the amount of both fractions in plasma of cirrhotic patient—the difference in the γ-globulin values was more striking, being almost doubled in amount over the

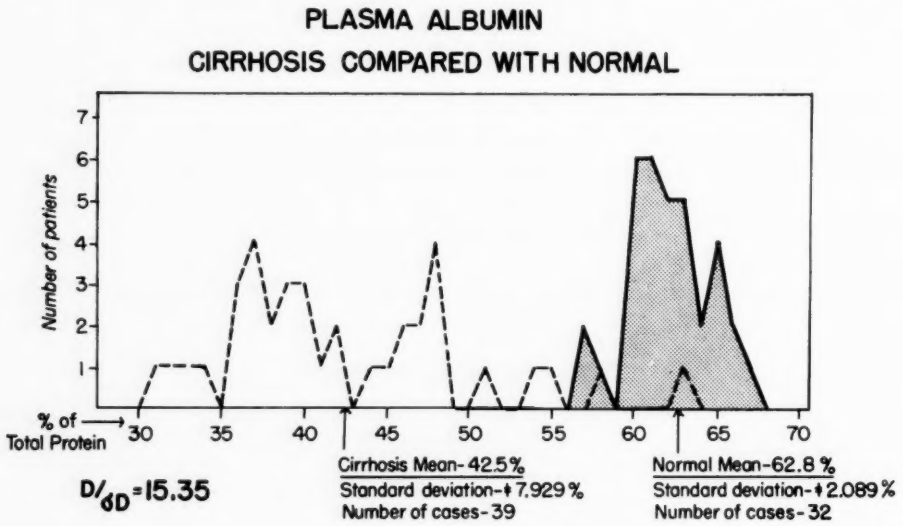

Fig. 2.

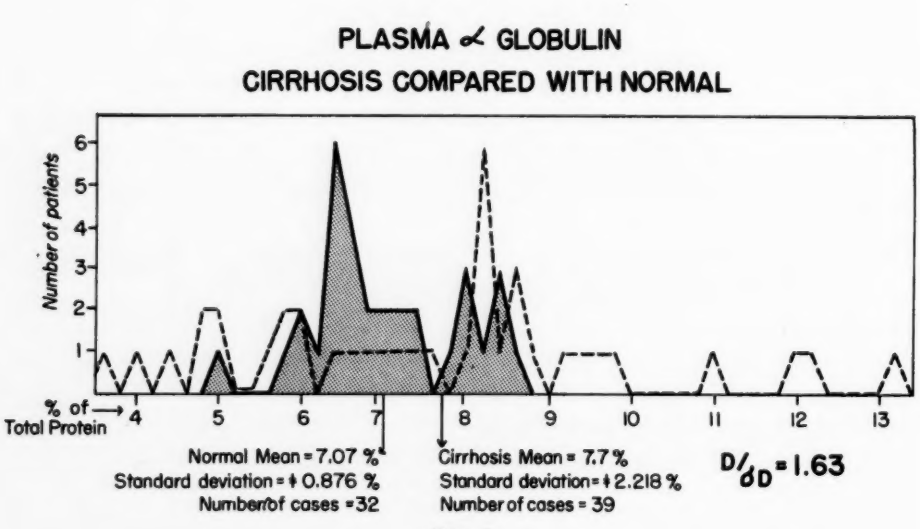
PLASMA α GLOBULIN
CIRRHOSIS COMPARED WITH NORMAL
Number of patients
% of Total Protein
Normal Mean = 7.07%
Standard deviation = ± 0.876%
Number of cases = 32
Cirrhosis Mean = 7.7%
Standard deviation = ± 2.218%
Number of cases = 39
D/σD = 1.63

Fig. 3.

normal averages. The degree of statistical significance of these β- and γ-globulin rises was calculated at 6.48 and 7.89 in grams per 100 ml. and 7.14 and 8.32 in per cent of total protein. (Figs. 4 and 5.)

There is good evidence [11, 12] that fibrinogen production largely depends on the liver, although other sites of formation have been implicated.[13, 14] A decrease in the plasma fibrinogen levels has been reported incident to liver damage

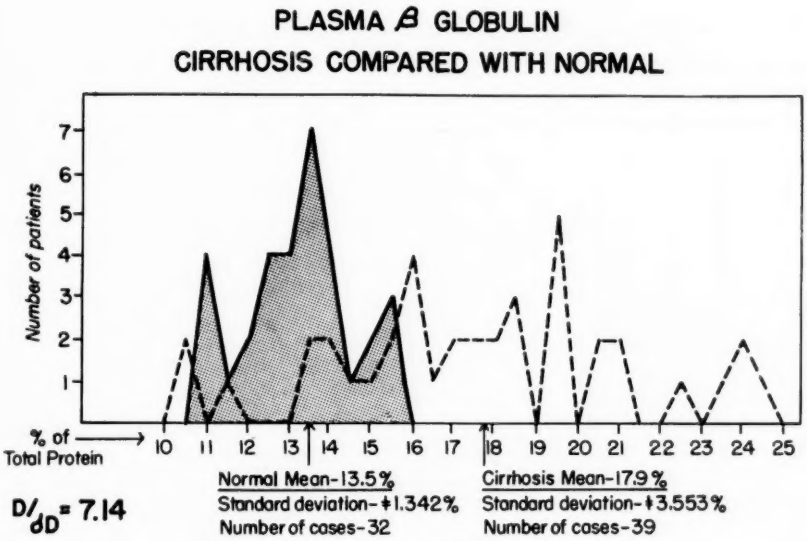

Fig. 4.

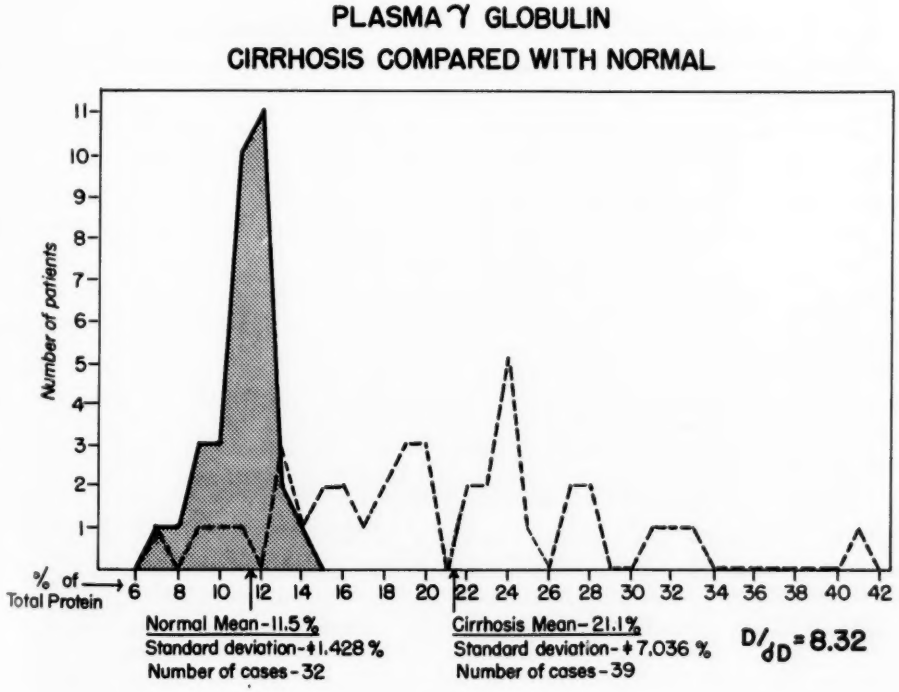

Fig. 5.

experimentally induced by carbon tetrachloride, phosphorus,[15] and chloroform,[16, 17, 18] and following hepatectomy.[19, 20] The fibrinogen content of the blood has been further correlated with the degree of liver injury.[21]

In contradistinction to these findings in experimental hepatic insufficiency in animals, there has been reported an increase of fibrinogen in the plasma of

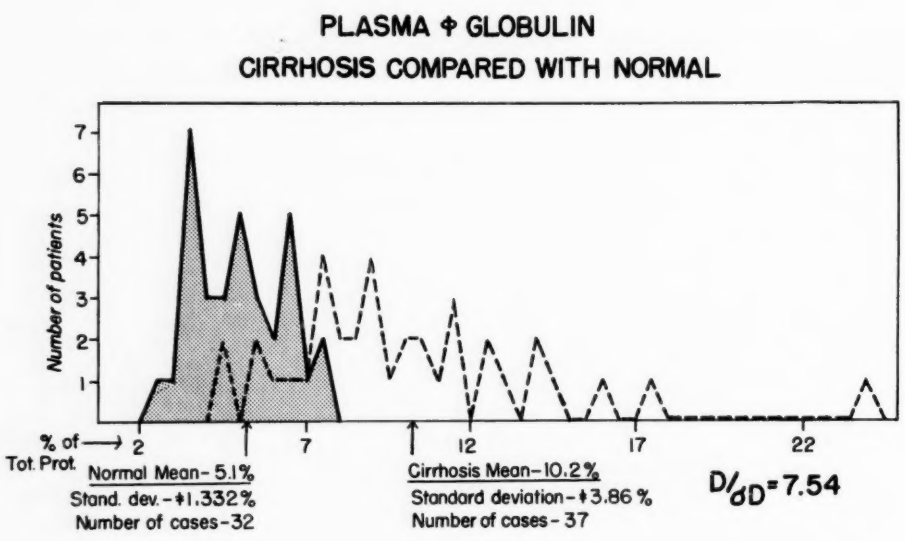

Fig. 6.

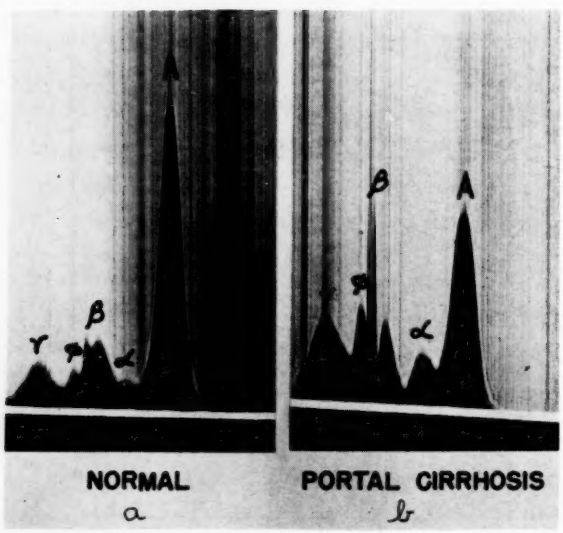

Fig. 7.—Demonstrating protein patterns in (*a*) a normal subject and (*b*) a cirrhotic patient whose fractions closely approximated the mean values for the group.

patients with portal cirrhosis[33] and our observations show a striking increase in the amount of the protein fraction having the mobility range of fibrinogen* in this disease. This rise is comparable in degree to the changes of β- and γ-globulin and is illustrated in Fig. 6. Most of the published work on the electro-

*Because γ_1-globulin, which has been concentrated from normal human plasma, has the same electrophoretic mobility as fibrinogen, an increase in the relative concentration of this peak could conceivably be due to an increase in γ_1-globulin as well as to a fibrinogen rise. The γ_1-globulin normally represents only about 3 per cent of the total serum protein and is not a clearly defined peak. The term φ-globulin is used to indicate the electrophoretic peak representing all fractions in the mobility range common to fibrinogen and γ_1-globulin.

phoretically determined protein changes in liver disease has been observation of serum protein alteration, thus lacking a demonstration of protein in this mobility range which might be compared with ours.

Increased amounts of β- and γ-globulin and lowered albumin have become relatively commonly accepted as being part of the laboratory picture of portal cirrhosis. It appears, however, that all measured fractions except α-globulin are definitely altered in quantity, both relatively and absolutely, in this disease. The results of our analyses showed no distortion of the individual fraction peaks which might suggest the presence of an abnormal protein. Changes were interpreted as being simply quantitative and involving qualitatively normal protein fractions (Fig. 7, *a* and *b*).

The formation of protein-rich ascitic fluid in patients with cirrhosis is further evidence—whether cause or effect—of abnormal protein metabolism. Many investigators[22-25] have related ascites formation to hypoalbuminemia, but changes in other fractions have not been incriminated.

Of the thirty-nine cirrhotic patients, twenty-five (64 per cent) had physical findings indicating ascites. A comparison of the mean values of the plasma protein fractions in patients having cirrhosis and ascites with the same observations in patients with cirrhosis lacking evidence of ascites is presented in Table III. In the ascitic group most of the values are lower—the total protein and albumin rather definitely so, the mean value of albumin being 2.78 Gm. per 100 ml. or 80 per cent as much as the average for patients without ascites. The α- and β-globulins are also lower in the group with ascites but to a lesser extent. There is little variation in fibrinogen levels, but the γ-globulin level in the group with ascites is distinctly elevated, being 45 per cent higher than in the non-ascitic group.

Protein fractions were electrophoretically calculated on the ascitic fluid from four patients. The results, listed in Table IV, were widely variable. The ascitic fluid was relatively richer in albumin than plasma from the same patient in each instance, and the mean value (expressed in per cent of total protein) was 10 per cent greater than the mean for all patients with cirrhosis. There was relatively less α-, β-, and ϕ-globulin in ascitic fluid than in the respective patient's plasma or than the mean for all patients with cirrhosis. The γ-globulin means were approximately the same, but two patients had higher and two had lower γ-globulin in ascitic fluid than in plasma.

The lowered plasma albumin in patients with cirrhosis results in lower oncotic pressure, which may aid in the development of ascites. This albumin deficit is probably also the stimulus for abnormal increases in other protein fractions—most noticeably those in the mobility range of γ-globulin. The finding of a relatively richer albumin in all and increased γ-globulin in two of the four ascitic fluids analyzed and of relatively lower α-, β-, and ϕ-globulin in these specimens may be indicative of an elective protein inclusion based on the molecular weight of the protein components. Current estimates of the molecular weights indicate that they increase in the order: albumin, γ-globulin, α_1-globulin, α_2-globulin, fibrinogen, and β-globulin.[26]

TABLE III. COMPARISON OF THE AVERAGE VALUES OF PLASMA PROTEIN FRACTIONS IN CIRRHOTIC PATIENTS HAVING ASCITES WITH THOSE LACKING EVIDENCE OF ASCITES

	TOTAL PROTEIN (GM.)*	ALBUMIN		α-GLOBULIN		β-GLOBULIN		γ-GLOBULIN		ϕ-GLOBULIN	
		GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†
Cirrhosis with ascites (mean)	6.61	2.78	42.0	0.49	7.3	1.15	17.4	1.57	23.8	0.68	10.4
Cirrhosis without ascites (mean)	7.33	3.43	46.6	0.61	8.2	1.37	18.7	1.21	16.4	0.73	10.1

*Grams per 100 ml. plasma.
†Per cent of total plasma protein.

TABLE IV. PROTEIN FRACTIONS IN ASCITIC FLUID

PATIENT	TOTAL PROTEIN (GM.)*	ALBUMIN		α-GLOBULIN		β-GLOBULIN		γ-GLOBULIN		ϕ-GLOBULIN	
		GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†
1	1.29	0.59	45.5	0.07	5.1	0.2	15.9	0.29	22.6	0.14	10.9
2	1.34	0.72	53.5	0.05	3.8	0.2	15.0	0.37	27.7		
3	3.4	2.06	60.5	0.17	5.1	0.44	12.9	0.57	16.7	0.16	4.8
4	0.84	0.41	48.9	0.21	6.2	0.13	15.1	0.15	18.0	0.99	11.8
Means	1.72	0.95	52.1	0.12	5.05	0.24	14.7	0.35	21.3	0.13	9.17
PLASMA PROTEIN FRACTIONS IN THE SAME PATIENTS											
1	6.08	2.31	39.0	0.37	6.0	1.44	23.7	1.19	19.6	0.71	11.7
2	5.87	2.64	45.0	0.24	4.1	1.03	17.5	1.45	24.8	0.51	8.6
3	5.93	2.88	48.6	0.55	9.2	0.97	16.3	1.08	18.3	0.45	7.6
4	6.03	2.46	40.8	0.52	8.7	0.98	16.2	1.21	20.1	0.86	14.2

*Grams per 100 ml. plasma.
†Per cent of total plasma protein.

There has been extensive experimental research into the relationship of specific protein fraction alteration with the results of some of our more recent liver function tests. Our observations include the attempt to determine possible reciprocity between plasma protein changes and the results of the thymol turbidity reaction, the cephalin-cholesterol flocculation test, and the bromsulfalein excretion test. The correlation with the prothrombin concentration and the degree of icterus was also analyzed.

Much diversity of opinion, and even of laboratory evidence, exists concerning the protein fractions which may be involved in the thymol turbidity reaction and of the effects of certain aspects of lipid metabolism in the test. Maclagan[27] in 1944 incriminated γ-globulin; Hanger and associates[28] held that the reaction was more dependent on the α- and β-globulin fractions; Kunkel and Hoagland[29] presented data which argued for γ-globulin responsibility; and Cohen[30] presented convincing evidence identifying β-globulin as the important fraction.

The thymol turbidity reaction was determined in twenty-two patients. Findings of 4 units or more were considered positive and were found in eleven patients—precisely 50 per cent. Mean values for the protein fractions of the eleven patients having positive tests are compared with the eleven negative reactors in Table V. Somewhat higher total protein and lower albumin values were found in the positive group. The calculations for α- and ϕ-globulin were very similar in the two groups, and the β-globulin was slightly increased in the group with positive reactions. The most striking difference is in the γ-globulin levels where the mean of the positive group exceeds that of the negative by more than 40 per cent. The significance of this observation has not been further investigated.

Comparing the mean values of protein fractions of patients who had cephalin-cholesterol flocculation reactions of 1 unit or less with those having reactions of 2 or more units results in a conclusion similar to the analysis of the thymol reactions. Eighteen patients were tested, nine having reactions interpreted as negative and nine having positive results. As shown in Table VI, there is very little difference in total protein, α-, β-, and ϕ-globulin. The significant observations seem to be a moderate decrease in the amount of albumin and a distinct increase in the γ-globulin in the group with positive tests. There is approximately 22 per cent more albumin in the negative group and 80 per cent more γ-globulin in the cephalin-cholesterol flocculation positive reactions. It appears that the decrease in albumin and the, perhaps compensatory, increase in γ-globulin are the changes most apt to be responsible for the cephalin-cholesterol flocculation reaction, as has been previously implied by some investigators.[31]

It is incidentally interesting to observe that three patients with positive thymol reactions had negative cephalin-cholesterol flocculations and two had positive cephalin-cholesterol flocculations who had negative thymol results. There were seven patients in whom both tests were positive and six in whom both were negative.

Bromsulfalein excretion was observed in thirteen patients with icteric indices less than 10 units. Twelve of these patients had bromsulfalein retention in excess of 10 per cent. There were no sifinificant differences between the

TABLE V. CORRELATION OF PLASMA PROTEIN CHANGES WITH THE THYMOL TURBIDITY REACTIONS

	TOTAL PROTEIN (GM.)*	ALBUMIN		α-GLOBULIN		β-GLOBULIN		γ-GLOBULIN		ø-GLOBULIN	
		GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†
Mean values for patients having thymol turbidity reactions of less than 4 units	7.13	3.53	49.6	0.60	8.4	1.23	17.2	1.15	16.2	0.67	9.4
Mean values for patients having thymol turbidity reactions of 4 or more units	7.59	3.25	42.5	0.60	7.8	1.34	17.7	1.73	22.7	0.70	9.3

*Grams per 100 ml. plasma.
†Per cent of total plasma protein.

TABLE VI. CORRELATION OF PLASMA PROTEIN CHANGES WITH CEPHALIN-CHOLESTEROL FLOCCULATION REACTIONS

	TOTAL PROTEIN (GM.)*	ALBUMIN		α-GLOBULIN		β-GLOBULIN		γ-GLOBULIN		ø-GLOBULIN	
		GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†
Mean values for patients having cephalin-cholesterol flocculation reactions of 1 unit or less	7.42	3.64	49.5	0.61	8.3	1.35	18.1	1.18	15.5	0.64	8.7
Mean values for patients having cephalin-cholesterol flocculation reactions of 2 or more units	7.37	3.03	40.5	0.62	8.4	1.28	17.4	1.8	24.6	0.76	10.4

*Grams per 100 ml. plasma.
†Per cent of total plasma protein.

TABLE VII. CORRELATION OF PLASMA PROTEIN CHANGES WITH DEGREE OF ICTERUS

	TOTAL PROTEIN (GM.)*	ALBUMIN		α-GLOBULIN		β-GLOBULIN		γ-GLOBULIN		ϕ-GLOBULIN	
		GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†
Mean values for patients with icteric indices less than 4	7.31	2.92	41.0	0.70	9.37	1.24	17.0	1.48	21.3	0.87	11.3
Mean values for patients with icteric indices more than 4	7.0	2.99	42.9	0.52	7.37	1.20	17.4	1.65	22.8	0.68	10.2
Mean values for patients with icteric indices more than 25	7.15	2.90	41.6	0.55	7.81	1.13	16.3	1.83	23.5	0.83	11.7

*Grams per 100 ml. plasma.
†Per cent of total plasma protein.

TABLE VIII. CORRELATION OF PLASMA PROTEIN CHANGES WITH THE PROTHROMBIN CONCENTRATION

	TOTAL PROTEIN (GM.)*	ALBUMIN		α-GLOBULIN		β-GLOBULIN		γ-GLOBULIN		ϕ-GLOBULIN	
		GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†	GM.*	%†
Mean values for patients having a prothrombin concentration of 86% of normal or more	7.27	3.23	44.6	0.61	8.33	1.39	18.9	1.43	19.9	0.66	9.1
Mean values for patients having a prothrombin concentration of less than 86%	6.90	2.77	40.9	0.48	7.0	1.13	16.9	1.76	24.0	0.76	11.2
Mean values for patients having a prothrombin concentration of less than 68%	6.97	2.68	39.7	0.48	6.9	1.15	17.2	1.92	25.1	0.78	11.2

*Grams per 100 ml. plasma.
†Per cent of total plasma protein.

mean protein values of these twelve patients and the means of the whole cirrhosis group. Since the performance of this test depends on the excretory function of the liver cells and the activity of the reticuloendothelial system,[32] there is little reason to expect protein changes to be involved primarily.

The presence of jaundice does not seem to alter protein significantly. There were only three patients who had icteric indices of less than 4 units, while thirty-four had higher indices. Fourteen patients had an index of more than 25 units. Table VII summarizes the mean values of the protein fractions of each of the three groups with varying degrees of jaundice. This lack of effect of icterus on serum proteins has been observed in patients with obstructive jaundice.[2]

The changes in quantity of the protein fractions incident to decreasing prothrombin concentrations are shown in Table VIII. These alternations may simply reflect the effect of increased liver damage and have little direct reference to the amount of prothrombin present. There are rather severe decreases in albumin and increases in γ-globulin as the prothrombin concentration decreases; the decrease in α- and β-globulin and increase in ϕ-globulin in the group with lower prothrombin concentration are less noticeable.

DISCUSSION AND SUMMARY

The scope of the long-noted clinical and laboratory evidence of the effect of liver disease on protein metabolism was notably enhanced by the introduction of the Tiselius apparatus in 1937. We have used this method to study the plasma and ascitic fluid proteins of patients with portal cirrhosis in an attempt to add more specific information about the alterations which result from this pathology and to relate these changes to the results of certain liver function tests which appear to be dependent on rather characteristic changes.

1. A comparison of the plasma protein fractions of thirty-nine patients having cirrhosis with those of thirty-two normal subjects revealed statistically significant differences for all fractions except the α-globulin. The albumin was decreased while β-, γ-, and ϕ-globulins were increased.

2. Plasma from patients with ascites contained 80 per cent as much albumin and 45 per cent more γ-globulin than plasma from cirrhotic patients without ascites. Changes in other fractions were less pronounced.

3. Ascitic fluid which was electrophoretically analyzed contained relatively more albumin than the same patient's plasma.

4. There was 40 per cent more γ-globulin in the plasma from eleven patients with positive thymol turbidity tests than in that from the eleven patients having negative reactions.

5. Nine patients with positive cephalin flocculation reactions had γ-globulin mean values 80 per cent higher than those of the group of nine with negative tests. The mean for albumin was 22 per cent higher in the negative group.

6. The presence of jaundice did not seem to influence protein change.

7. Lower albumin and higher γ-globulin levels were associated with depressed prothrombin concentration.

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SYSTEMIC AND PORTAL VENOUS PRESSURES IN CIRRHOSIS OF THE LIVER

CHARLES S. DAVIDSON, M.D., THOMAS B. GIBBONS, M.D., AND WILLIAM W. FALOON, M.D.
BOSTON, MASS.

IN SPITE of considerable investigation of the subject, the mechanism of the formation of ascites and edema in patients with cirrhosis of the liver has not been fully elucidated. Among the factors considered have been (a) diminished plasma colloid osmotic pressure due to a lowered plasma albumin concentration,[1] (b) altered permeability of the capillaries, (c) failure to excrete sodium and water in the urine in a normal manner,[2, 3] and (d) increase in the venous pressure in the inferior vena cava and portal vein.[4, 5] There is a paucity of data concerning the venous pressure in various locations of the body in cirrhosis of the liver, particularly in the portal vein.

In the present study, direct measurements of the pressure in antecubital, femoral, and anterior abdominal veins were correlated with the extent of ascites and edema. In addition, the effect of abdominal paracentesis on the pressure in these veins was noted. A method for the indirect measurement of approximate portal vein pressure is presented.

CLINICAL MATERIAL AND METHODS

Ten patients with chronic hepatic cirrhosis (alcoholic) were studied, eight with ascites and seven with edema. All had clear-cut clinical and laboratory evidence of severe liver disease. In several instances the diagnosis was substantiated by punch biopsy of the liver. In addition, one patient with a "chocolate" cyst of the ovary containing over 5 liters of fluid was studied. Direct venous pressures were determined with the patient supine, using the phlebomanometer described by Winsor and Burch, and the precautions of technique they described were heeded.[6] The "phlebostatic axis," lying on a frontal plane passing midway between the anterior and posterior surfaces of the thorax, was the reference level to which all measurements were corrected. To ascertain the ascitic fluid pressure a 20 gauge needle was inserted directly into the peritoneal cavity at its most ventral point with the patient lying on his back. Venous pressure was measured in an antecubital and a femoral vein and also in an anterior superior abdominal vein. A second reading was obtained in the anterior abdominal vein after manually compressing it proximal (cephalad) to the site of insertion of the needle. It is believed that the pressure obtained by this maneuver is close to that in the portal vein. The reasons for this assumption will be presented below.

Serum albumin concentration was determined after fractionation of the serum protein by the method of Howe.[7]

RESULTS

Antecubital Vein Pressure.—As may be seen in Table I, the antecubital venous pressure varied from 95 to 176 mm. of water. The normal range for

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School.

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pressure in this vein with the method used is 50 to 140 mm. of water. Antecubital vein pressures in three of the ten measurements in the patients with cirrhosis were slightly elevated.

Femoral Vein Pressure.—The femoral venous pressure was elevated in all but one of the patients studied. The patient with the normal reading had neither ascites nor edema. In the eight patients with ascites it was markedly elevated, usually as high as or higher than the ascitic fluid pressure. Edema of the legs usually was most marked in the patients with the highest femoral venous pressure, although three patients with definitely elevated femoral pressures had no discernible edema of the lower extremities.

Collateral Venous Pressure—Free Flow.—The pressures in the superior abdominal collateral veins are also listed in Table I. It is readily seen that the

TABLE I

SUBJECT	SERUM ALBUMIN (GM./100 C.C.)	INTERVAL BETWEEN PARACENTESES (DAYS)	VENOUS PRESSURE (MM. WATER)				ASCITIC FLUID PRESSURE (MM. WATER)	ASCITES (0 TO 4+)	EDEMA (0 TO 4+)
			ANTECUBITAL	FEMORAL	COLLATERAL				
					FREE FLOW	COMPRESSED CEPHALAD			
C. M.	3.4	21-28	110	180	210	240	180	3+	0
H. F.	2.4	45	147	302	213	255	270	4+	4+
M. C.	2.6	18	115	280	205	285	280	4+	2+
A. D.	3.0	∞	130	180	245	300	–	0	4+
J. F.	–	∞	95	85	165	315	–	0	0
M. B.	3.4	14	115	249	135	385	185	3+	1+
J. W.	3.4	20	130	295	310	395	235	4+	3+
J. W.*	4.9	10	120	368	390	463	400	4+	3+
R. K.	–	7-12	176	178	172	400	186	3+	0
C. D.	–	∞	135	237	225	–	–	2+	3+
J. K.	–	–	155	268	–	–	260	4+	2+
A. M.†	–	–	155	335	175	335	250 (cyst)	4+ (cyst)	0

*Second examination, following the intravenous administration of human serum albumin.
†Patient with ovarian cyst.

pressure in these veins, when corrected to the reference level, varied considerably from patient to patient. In all but one instance this pressure exceeded that in the antecubital vein. Generally speaking, however, these pressures were somewhat below the range usually accepted for portal vein pressure in patients with cirrhosis of the liver.[8-12]

Collateral Venous Pressure With Proximal Compression of the Veins.—When the collateral vein in which venous pressure was being measured was compressed proximal to the site of insertion of the needle (i.e., nearer the heart), a considerable rise in the pressure occurred in all instances. The venous pressure after such compression was as high as or considerably higher than the ascitic (or cyst) fluid pressure in all instances. This would indicate that the obstructed collateral pressure was closer to a measurement of portal vein pressure than that observed when the flow of blood was free, since the pressure of blood in the portal vein supplying the collaterals on the anterior abdominal

wall must be at least as high as that of the ascitic fluid or the veins would be compressed, preventing the flow of blood. Furthermore, the pressures recorded after the collateral had been proximally compressed approximate those recorded in the literature from direct measurements of the portal vein pressure.[8-12]

Ascitic Fluid Pressure and Rate of Ascites Formation.—The pressure of the ascitic fluid for each of seven patients with ascites is shown in Table I. In the table the patients with cirrhosis are listed in order of increasing compressed collateral venus pressure. The patients in whom this pressure was highest had

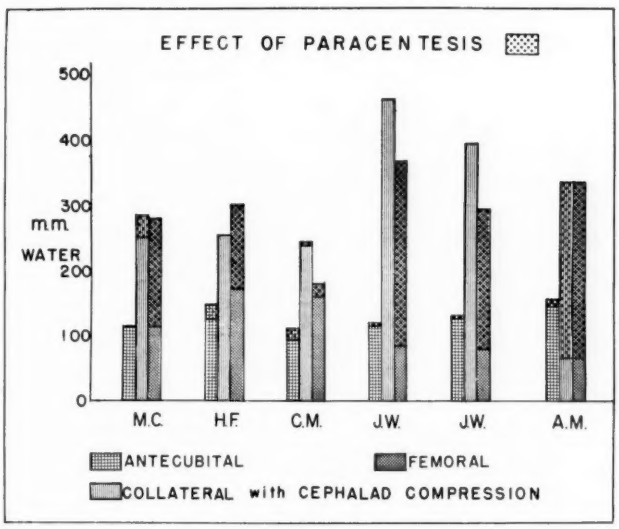

Fig. 1.

relatively short intervals between paracenteses, although this relationship was not an absolute one. Thus, the two patients (Subjects A. D. and J. F.) who were not forming ascites had higher obstructed collateral vein pressure than two others (Subjects H. F. and M. C.) with massive ascites which had reformed in eighteen and forty-five days.

The serum albumin concentration, measured in six patients, was low except in one patient, Subject J. W., to whom albumin had been administered intravenously before the second series of measurements. There was no correlation between the serum albumin concentration and the interval between paracenteses, although the series is too small to be definitive.

Effect of Paracentesis.—In Fig. 1 the measurements are shown for venous pressures in the antecubital, femoral, and collateral veins (with proximal compression) before and after paracentesis. The antecubital vein pressure fell only very slightly or not at all following removal of ascitic fluid. This was true also of the pressure in the obstructed collateral vein in the four patients with cirrhosis and ascites. In the patient, Subject A. M., whose abdominal swelling was due to a large cyst of the ovary, and who, by clinical examination and

laboratory studies, demonstrated no evidence of primary liver disease, the venous pressure response in the superior abdominal vein was different. In her case there was no obstruction to the flow of portal venous blood other than that due to the cyst so that when the cyst fluid was removed the venous pressure in the superior abdominal vein fell to less than 100 mm. of water despite proximal manual obstruction. The contrast between the effect of paracentesis on the superior abdominal venous pressure in this patient and in the patients with hepatic disease again suggests that the pressure measured in the abdominal collateral vein when compressed proximally was closely related to the portal venous pressure itself.

Following paracentesis, the femoral venous pressure fell to normal or near normal in all patients. This might be expected since with ascites present the pressure in the inferior vena cava and its tributaries must be raised considerably to permit flow of blood to the heart.

DISCUSSION

In 1937 Ferris and Wilkins[13] in this laboratory reported on the diagnostic value of comparative measurements of the pressure in the antecubital and femoral veins. Case 7 reported by them concerned a patient with cirrhosis of the liver with ascites and edema. They noted that whereas the pressure in the antecubital vein was not affected by paracentesis, the femoral venous pressure fell from between 170 and 245 mm. of water to between 100 and 120 mm. of water after paracentesis. They concluded that the femoral venous pressure was directly related to the ascitic fluid pressure. These authors indicated that there were two factors in the maintenance of the pressure in the ascitic fluid, as reflected by the rapidity of fall of the femoral venous pressure. As soon as paracentesis was begun and the first small volume of ascites released, a pronounced fall in the femoral venous pressure occurred, presumably related to the tension of the ascites in the peritoneal cavity. After this striking drop, the femoral venous pressure gradually fell at a rate equal to that of the fall of the ascitic fluid pressure measured by a manometer attached to the paracentesis cannula. They attributed this second, slower rate of fall to the decrease in the hydrostatic pressure of the ascites. The results of the present study confirm those of Ferris and Wilkins and also are similar to those of Levy and Burch[14] who described reductions in the pedal venous pressure after paracentesis in patients with hepatic cirrhosis. The clinical observation that edema of the lower extremities often clears promptly following paracentesis would appear to be related to the fall in femoral venous pressure, although this is undoubtedly not the only factor controlling edema formation in the lower extremities. In the studies reported here there appeared to be a rough correlation between the femoral venous pressure and the occurrence of dependent edema. However, three patients with definitely elevated femoral pressure had no clinically detectable edema of the feet or legs. This finding has been reported in patients after ligation of the inferior vena cava.[15]

When blood is allowed to move freely through the collateral venous channels of the anterior abdominal wall, the pressure in these veins must be the algebraic sum of two forces: the positive portal pressure and the negative intrathoracic pressure. Thus, one would expect a progressive drop in pressure to occur as the blood moves from the portal vein itself to the entrance of the collateral veins into the chest. For this reason the pressure measured in the anterior abdominal veins when a free flow of blood is permitted could not be as great as the portal pressure itself.

When the blood in these collateral veins was prevented from flowing freely toward the heart, a moderate (30 mm.) to marked (228 mm.) increase in pressure was found. This maneuver brought the collateral venous pressure up to or above the ascitic fluid pressure in every instance, whereas when free flow was allowed the collateral pressure was often below the ascitic fluid pressure. It can be considered, therefore, that the pressure obtained with obstructed flow was nearer to the portal pressure than that measured when free flow was permitted, since the portal pressure must be at least as high as the ascitic fluid pressure to allow flow of blood through the portal vein channels. Another indication that the obstructed collateral pressure was related to the portal venous pressure was the failure of the former to fall following paracentesis whereas that in the femoral vein dropped abruptly. Thus, in the case of the portal vein, the obstruction was not relieved by removal of the ascitic fluid. This fact was further confirmed by the sharp fall in the pressure in the obstructed collateral vein in the patient with the ovarian cyst but with a normal liver, the obstruction to the portal flow in this instance having been the cyst itself. Finally, the pressure readings obtained in the superior abdominal collateral veins when the blood flow in them was blocked compare closely with published portal vein pressures measured at the time of laparotomy by insertion of a needle into the portal vein itself.[8-12]

From the observations presented it is not possible to decide the relative importance of the factors which have been implicated in the formation of ascites and edema in cirrhosis of the liver. The results indicate, however, that neither the increased femoral nor portal venous pressures could account alone for the formation and maintenance, respectively, of edema and ascites, nor was there a correlation with the colloid osmotic pressure as determined by the serum albumin concentration. In this regard studies of the serum albumin concentration and of its artificial change by the administration of normal human serum albumin to patients with cirrhosis of the liver have not implicated the colloid osmotic pressure of the plasma as the primary factor in the cause of edema and ascites.[16, 17]

No attempt has been made to investigate the permeability of the capillaries in the peritoneum or in the lower extremities of patients with cirrhosis of the liver. It is possible that this factor may be important in the maintenance of ascites and edema.

Finally, the failure to excrete sodium and water in normal quantities which has been recently demonstrated in patients with cirrhosis of the liver[2, 3] probably contributes in some measure to the tendency to ascites and edema formation.

SUMMARY

1. The blood pressure was measured in the antecubital, femoral, and superior abdominal collateral veins of ten patients with chronic cirrhosis of the liver (alcoholic) and of one patient with a large ovarian cyst. When ascites was present, ascitic fluid pressure was measured. The pressure in the ovarian cyst likewise was measured.

2. The antecubital venous pressure was within the normal range in seven and slightly elevated in four of the patients studied.

3. The femoral venous pressure was usually as high as or higher than the abdominal fluid pressure. The presence and extent of edema of the lower extremities roughly correlated with the femoral vein and abdominal fluid pressures in nine of the eleven patients.

4. The abdominal collateral vein pressure measured when free flow of blood through the vein was permitted was usually below the ascitic fluid pressure.

5. In the eleven patients with cirrhosis the pressure in an abdominal collateral vein when measured with proximal manual obstruction to the blood flow was as high as or higher than the ascitic fluid pressure, remained elevated after paracentesis, and was in the same range as the direct portal vein pressures obtained by others at operation. Likewise, this pressure was elevated in the patient with the ovarian cyst, but fell promptly following drainage of the cyst fluid. It is concluded that the collateral pressure measured in this way approaches the true portal vein pressure.

6. The obstructed collateral vein pressure was generally highest in the patients with the shortest interval between paracenteses, although in two patients not forming ascites this pressure was likewise elevated.

7. The serum albumin concentration did not correlate with the ascitic fluid pressure nor with the rate of ascites formation.

8. The increased femoral and portal vein pressures are a measurable factor but cannot alone account for the formation and maintenance of edema or ascites in patients with cirrhosis of the liver.

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IN VITRO SENSITIVITY OF COLIFORM BACILLI TO SEVEN ANTIBIOTICS (PENICILLIN, STREPTOMYCIN, BACITRACIN, POLYMYXIN, AEROSPORIN, AUREOMYCIN, AND CHLOROMYCETIN)

PAUL F. FRANK, M.S., CLARE WILCOX, AND MAXWELL FINLAND, M.D.
BOSTON, MASS.

IN SPITE of the wide range of activity of the effective antibiotics that are now in use, it is well recognized that microorganisms vary considerably in their susceptibility to these agents. Not only do individual species of organisms vary in their susceptibility to different antibiotics, but also the different species vary markedly in their sensitivity to the same antibiotic. Moreover, different strains of the same species may exhibit wide variations in their sensitivity to the same antibiotic. In general, the sensitivity or resistance of any species or strain of organism to any one antibiotic is quite independent of its susceptibility to any other antimicrobial agent, except, of course, in the case of chemically closely related agents, like the various penicillins.

The in vitro sensitivity of bacteria to any given antibiotic agent has served as a useful guide to the value of that agent in the therapy of infections with the corresponding bacterial species. The limitations of this generalization, however, are well recognized and are exemplified in the case of typhoid fever which is not favorably influenced by streptomycin although the causative organisms may be sensitive in vitro to concentrations which are readily attained in the blood and other body fluids. Many factors such as absorption, distribution, and excretion of the antibiotic, its plasma-binding properties, toxicity, and tendency to select out resistant variants all play a part in determining the therapeutic effectiveness of that antibiotic in infections with organisms that are apparently susceptible in vitro. Likewise, the conditions under which the in vitro tests for sensitivity are performed must also be considered since they may profoundly influence the results of the tests, and the optimum conditions for such tests may vary for the different antibiotic agents and for different species of organisms.

The present series of studies were designed primarily to make direct comparisons of the effectiveness of a group of antibiotics against a variety of common species of pathogenic aerobic bacteria insofar as that is possible from in vitro tests. In the case of each species of organism, tests were carried out simultaneously with a number of recently isolated strains and with all of the antibiotics under study. In this way it was possible to obtain valid comparisons of the susceptibility of each strain to the different antibiotics and at the same time to compare the sensitivity of different strains to each of the antibiotics used. In the case of the antibiotics which were being used clinically, an appreciably larger

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital and the Department of Medicine, Harvard Medical School.
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number of strains were tested for their sensitivity to these agents at the time the strains were isolated from infections in which the antibiotics were to be used. The results of these tests, when considered in conjunction with those of the necessarily small groups of organisms that were specifically included for the comparative studies, provide a more reliable picture of the range of sensitivity of different strains of the same organisms to these clinically important antibiotics.

It has been found most convenient to present the results of the studies separately for each of the bacterial species. The present paper includes the results obtained with the most common of the pathogenic coliform bacilli, namely *Escherichia coli*, *Aerobacter aerogenes*, and *Klebsiella pneumoniae*. The results of the tests with other pathogenic bacteria will be presented in subsequent papers of this series.

MATERIALS AND METHODS

Sources of the Strains.—The materials from which the strains of bacteria used in this study were cultured were all obtained from patients with active infectious processes. With few possible exceptions these organisms were isolated from sources which indicated that they probably were significantly concerned in the infections. The source materials are listed in Table I. While strains of organisms isolated from patients after antibiotic therapy carried out in this hospital were deliberately excluded from the present study, it is highly probable that a small number of the patients had received some form of antibiotic therapy before the material was obtained that yielded some of these strains, particularly some of those isolated in recent months.

The majority of the strains were obtained in pure culture from blood or other body fluids and most of the others represent the predominant organisms in the cultures from which they were isolated. They were identified by their morphologic, colonial, and biochemical characteristics. (Most of the strains were isolated and identified in the Bacteriological Laboratory of the Mallory Institute of Pathology by Marion E. Lamb and A. Kathleen Daly; a few were received from other hospital laboratories and the remainder were isolated and identified in this laboratory with the assistance of Janice M. Bryan.) The pure cultures were maintained by frequent subculture in brain-heart infusion broth (Difco), pH 7.4±, until ready for the tests.

TABLE I. SOURCES OF THE STRAINS TESTED*

SOURCE	ESCH. COLI		A. AEROGENES		K. PNEUMONIAE	
	NUMBER	%	NUMBER	%	NUMBER	%
Blood	29	10	31	9	21	18
Urine	202	69	229	69	37	32
Exudates	17	6	11	3	10	9
Wounds, abscesses	30	10	17	5	3	3
Sputum, pharynx	8	3	39	12	44	38
Feces	5	2	3	1	--	--
Total	291	100	330	100	115†	100

*Includes only strains the sources of which were known and recorded.

†Fifty-one of the strains were successfully classified into serologic types (Julianelle) as follows: Type A, 36 strains; Type B, 4 strains; Type C, 7 strains; and Type D, 4 strains.

Antibiotics.—All of the antibiotics except chloromycetin were available in dry form in sterile, rubber-capped vials. For the special tests in which the antibiotics were compared, they were all freshly diluted just prior to use by adding 10 or 20 ml. of broth to the vial and further dilutions were then made in broth. In the usual "clinical" tests, when only one or two antibiotics were used with a small number of strains, a desired amount was withdrawn from the vial for these tests and the remainder was immediately stored in the

frozen state at -25° C. for further use in subsequent tests. The chloromycetin was weighed out and dissolved in an appropriate amount of broth which was then heated at 100° C. for fifteen minutes before being used in the tests.

The following antibiotics were used: (1) *Crystalline potassium penicillin G*, potency 1,550 units per milligram, prepared by Commercial Solvents Corporation. The dilutions were made in terms of units and converted into micrograms (1 unit = 0.6 γ). (2) *Streptomycin hydrochloride* (Merck) was used in the early clinical tests and *streptomycin calcium chloride complex* (Merck) was used exclusively during the last two years and in all of the comparative tests. Dilutions were prepared on the basis of micrograms of the streptomycin base. (3) *Bacitracin* was provided by Commercial Solvents Corporation through the courtesy of Dr. Lawrence W. Smith. The preparation used throughout this study assayed at 34 units per milligram and the dilutions were prepared on the basis of the dry weight of the starting material. (4) *Polymyxin hydrochloride* (B71, Lederle) was supplied by Dr. Stanton M. Hardy in vials containing 20 mg. each. (5) *Aerosporin* (Polymyxin B) was supplied by Chas. Pfizer & Co., Inc., in 40 mg. vials through the courtesy of Dr. Gladys L. Hobby. (6) *Aureomycin hydrochloride* was available in crystalline form in vials containing 20 or 100 mg. each and was also furnished by the Lederle Laboratories through the courtesy of Dr. Hardy. (7) *Chloromycetin* (crystalline chloramphenicol) was provided by Dr. E. A. Sharp, Director of the Department of Clinical Investigation, Parke, Davis & Company.

Tests for Sensitivity.—Two methods were used in the tests for sensitivity of the strains to the various antibiotics. As a routine in the clinical testing of organisms for the selection or control of therapy, the *tube dilution method* of Rammelkamp and Maxon[1] was used with minor modifications. A suitable range of twofold dilutions of the antibiotic in 0.5 ml. amounts of broth was set up in 11 by 100 mm. culture tubes. To each tube was added 0.5 ml. of a 10^{-4} dilution of a fully grown culture of the test organism in broth containing 0.1 per cent defibrinated horse blood, the latter to serve as an indicator of growth. The mixture was then incubated for eighteen hours and visible growth noted. Subcultures to antibiotic-free heart infusion agar (Difco), pH 7.4±, were then made from the tubes showing either partial growth or no growth. The end point for complete inhibition (M.I.C., minimum concentration required for complete inhibition) was considered to be the smallest final concentration in which no growth occurred after twenty-four hours of incubation on the agar. The end point for partial inhibition was considered to be the lowest concentration in which either faintly visible growth was observed in the tubes or in which any growth occurred on the agar after transplantation from tubes which showed no discernible growth.

The *plate dilution method* was used in all of the comparative tests when a number of strains of the same organism were tested with all of the antibiotics simultaneously. During the past year this method also was used whenever there were several strains of the same or related species to be tested simultaneously with any given antibiotic for clinical purposes. In this test, suitable twofold dilutions of the antibiotics were made in heart infusion agar (Difco), pH 7.4±. The agar plates were each divided into six or eight segments and the fully grown cultures were each streaked within these segments by means of a loop of 2 mm. diameter. The plates were then inspected for growth with the aid of a hand lens (3×) after twenty-four and again after forty-eight hours of incubation and the extent of growth was recorded. The end point for complete inhibition for these tests was taken as the lowest final concentration of antibiotic on which there was no discernible growth after forty-eight hours. The end point for partial inhibition was considered to be the lowest concentration either on which growth was not discerned until forty-eight hours or on which there was much less growth as compared with the growth of the same strain on antibiotic-free agar.

Controls for the growth of the organisms on antibiotic-free media were included each time as were also tests with standard reference strains of known sensitivity.

In many comparative tests there was close agreement between the results obtained when the same organisms were tested simultaneously by these two methods. When differences did occur, the end point in the plate dilution was usually at a concentration that was twice that obtained by the tube dilution method. In addition, partial inhibition was demon-

strated more frequently over a range of 1 to 3 dilutions, that is, the difference between complete and partial inhibition may be two- to eightfold in the plate dilution method whereas the tube dilution method usually gave a sharp end point, or partial inhibition in only one dilution beyond the complete inhibiting end point.

RESULTS

The results of the tests will be presented separately for each of the bacterial species. In each instance the comparative tests that were carried out simultaneously with a group of unselected strains and with all of the antibiotics will be presented first. The data for all of the strains that were tested with each antibiotic will then be summarized and compared.

Escherichia coli.—The results of the tests for sensitivity of twenty-two recently isolated strains carried out by the plate dilution method are shown in Table II.* All of the concentrations have been expressed in micrograms per milliliter so that direct comparisons may readily be made on a weight basis. The end points are shown for both complete and partial inhibition according to the criteria mentioned. The difference between these values indicates the range of concentrations over which varying degrees of significant inhibition occurred.

Several points are readily apparent from these results. (1) There is a striking difference in the concentrations of the different antibiotics that are required to inhibit most of the strains. As little as 1 to 2 γ per milliliter are required for complete inhibition of most of the strains by polymyxin and aerosporin; about 6 γ per milliliter are required to produce a similar effect with chloromycetin or aureomycin; twice that amount is needed for most of the strains in the case of streptomycin, and as much as 150 γ per milliliter of penicillin are necessary to achieve the same effect on most of the strains. (2) There is a wide variation in the sensitivity of different strains to penicillin, ranging from 38 to more than 6,000 γ per milliliter; this range is only slightly narrower in the case of streptomycin, whereas each of the other antibiotics inhibits almost all of the strains within a narrow range of concentrations. (3) It is also seen that the sensitivity or resistance of any given strain to one antibiotic is independent of the effect of other antibiotics on that strain. This is well exemplified in the case of strains 2 and 18. (4) It is also of interest to note that the concentrations of penicillin required to inhibit some of these strains are not many times greater than the concentrations of streptomycin needed to inhibit most of them. (5) The spread between the concentrations of streptomycin required for complete and for partial inhibition was four- to eightfold for most strains as compared with a twofold difference in the case of the other antibiotics. Such a wide spread, however, was not observed in the tests done by the tube dilution method. In tests for streptomycin sensitivity carried out by the latter method on more than 150 strains of *Esch. coli*, 37 per cent gave a sharp end point (that is, full growth in the next dilution beyond that in which complete inhibition occurred) and 58 per cent of the strains showed partial inhibition in only a single dilution beyond the end point of complete inhibition.

*In this instance the tests were done simultaneously with only six antibiotics since chloromycetin was not made available to us until after this part of the test was completed. The test with chloromycetin was carried out later and some of the strains had been lost in the meantime.

TABLE II. SENSITIVITY OF TWENTY-TWO STRAINS OF ESCH. COLI TO SEVEN ANTIBIOTICS

STRAIN	SOURCE	PENICILLIN		STREPTOMYCIN		BACITRACIN		POLYMYXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
		C	P	C	P	C	P	C	P	C	P	C	P	C	P
1	Urine	150	75	25.0	3.1	10,000	5,000	1.0	—	1.0	0.5	6.3	3.1	6.3	3.1
2	Urine	>6,000	—	12.5	3.1	10,000	5,000	2.0	1.0	2.0	0.5	6.3	3.1	6.3	3.1
3	Urine	150	75	12.5	3.1	5,000	—	2.0	—	2.0	0.5	6.3	—	6.3	3.1
4	Blood	75	—	12.5	3.1	5,000	—	2.0	1.0	1.0	0.5	12.5	6.3	N.D.	
5	Kidney	75	—	12.5	3.1	5,000	—	4.0	2.0	1.0	0.5	25.0	6.3	12.5	3.1
6	Urine	300	75	12.5	3.1	5,000	2,500	2.0	1.0	1.0	0.5	6.3	3.1	6.3	3.1
7	Kidney	38	19	12.5	3.1	5,000	2,500	2.0	1.0	1.0	0.5	6.3	3.1	3.1	—
8	Kidney	150	39	25.0	3.1	5,000	2,500	2.0	1.0	1.0	0.5	6.3	—	6.3	3.1
9	Urine	150	75	12.5	3.1	5,000	—	2.0	—	1.0	0.5	12.5	3.1	6.3	—
10	Urine	150	75	12.5	3.1	5,000	—	2.0	1.0	2.0	0.5	6.3	3.1	6.3	3.1
11	Urine	150	75	12.5	3.1	5,000	—	1.0	—	1.0	0.5	6.3	3.1	N.D.	
12	Abscess	150	75	50.0	12.5	5,000	—	N.D.		10.0	1.0	12.5	3.1	N.D.	
13	Wound	150	75	12.5	3.1	5,000	—	1.0	—	1.0	0.5	12.5	3.1	N.D.	
14	Spinal fluid	3,000	600	6.3	3.1	10,000	—	10.0	1.0	1.0	0.5	3.1	—	3.1	—
15	Wound	75	—	12.5	3.1	5,000	2,500	2.0	1.0	1.0	0.25	6.3	3.1	6.3	3.1
16	Kidney	150	75	12.5	3.1	5,000	—	4.0	2.0	1.0	0.5	12.5	6.3	6.3	—
17	Blood	75	39	12.5	3.1	5,000	—	1.0	—	1.0	0.5	6.3	—	3.1	—
18	Urine	75	39	>1,000	—	10,000	5,000	1.0	—	1.0	0.5	6.3	3.1	6.3	3.1
19	Peritoneum	150	75	12.5	3.1	5,000	—	2.0	1.0	1.0	—	6.3	—	25.0	6.3
20	Urine	150	75	12.5	3.1	5,000	—	1.0	—	1.0	0.5	6.3	—	6.3	—
21	Urine	6,000	3,000	>1,000	—	5,000	—	1.0	0.5	1.0	0.5	25.0	6.3	N.D.	
22	Urine	150	75	12.5	1.6	5,000	—	2.0	1.0	1.0	0.5	25.0	12.5	6.3	—
98*	Stock	0.02	0.01	N.D.	N.D.	1.8	0.9	N.D.	N.D.	N.D.	N.D.	0.8	0.4	N.D.	N.D.
T†	Stock	N.D.	N.D.	1.6	0.8	N.D.	N.D.	0.5	0.3	1.0	0.5	N.D.	N.D.	1.6	0.8

All sensitivities expressed in γ per milliliter.

C, complete inhibition; P, partial inhibition (few discrete or many tiny colonies).

—, Full growth in next dilution; N.D., not done.

*Reference strain, Group A hemolytic streptococcus, strain 98.

†Reference strain, *K. pneumoniae*, Type A, strain T.

The distribution of the inhibiting concentrations of the various antibiotics for all of the strains of *Esch. coli* that have been tested is shown in Table III and is represented graphically in Fig. 1. Only the complete inhibiting concentrations are shown. The inclusion of the data for much larger numbers of strains in the case of the antibiotics that have been used clinically provides a more reliable picture of the range of sensitivity of this species to these antibiotics. In Fig. 1, the height of the peak and the spread of the distribution curve for each antibiotic indicate the degree of variation in the concentrations of that antibiotic

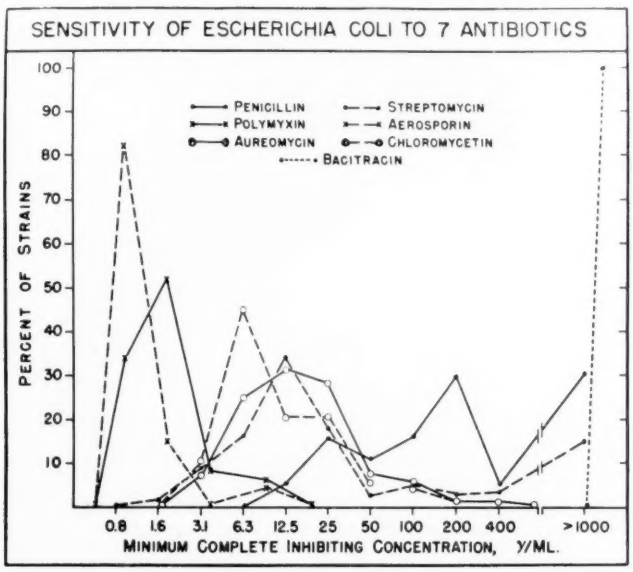

Fig. 1.

that are required to inhibit the different strains. These distribution curves exhibit considerable similarity over most of the range of concentrations in the case of streptomycin, chloromycetin, and aureomycin and are in contrast to high peaked curves with a narrow spread in the case of polymyxin, aerosporin, and bacitracin and the broad and irregular curve of penicillin. In the case of streptomycin, however, the curve has a second rise in high range of concentration corresponding to the resistant strains.

These comparisons are more readily visualized when the same data are charted with the ordinate representing the cumulative per cent of strains, as shown in the lower section of Fig. 2. This method of representing the data also avoids, or at least minimizes, the complexity of the figure in that the various distribution curves do not tend to cross frequently as they necessarily do in the normal type of distribution curves. In Fig. 2, the slope of each curve gives an indication of the range of concentrations required for different strains—the steeper the slope, the narrower the range. The position of the various curves from left to right in Fig. 2 provides a ready comparison of the activity of the different antibiotics, at least on a weight basis.

TABLE III. SENSITIVITY OF PATHOGENIC STRAINS OF ESCH. COLI TO SEVEN ANTIBIOTICS

M.I.C. (γ/ML.)	PENICILLIN		STREPTOMYCIN		BACITRACIN		POLYMYXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL
1-1.6	1	2	2	1.1			7	33	18	82	1	0.6		
2-3.1	0		10	5.3			11	52	3	14	7	4.1	4	8
4-6.3	0		30	15.8			2	10	0		41	23.8	23	45
10-12.5	2	5	64	33.7			1	5	1	4	53	30.8	10	20
25	7	17	34	17.9							47	27.3	10	20
38-50	5	12	4	2.1							14	8.1	2	4
75-100	7	17	8	4.2							7	4.1	2	4
150-200	13	31	2	1.1							1	0.6		
300-750	2	5	5	2.6							1	0.6		
> 1,000	5	12	31	16.3	22	100								
Total	42	100	190	100.	22	100	21	100	22	100	172	100.	51	100.

M.I.C., minimum concentration required for complete inhibition.

From Fig. 2 it is seen that the order of the in vitro activity of the seven antibiotics arranged on a weight basis is as follows: (1) aerosporin, (2) polymyxin, (3) chloromycetin, (4) aureomycin, (5) streptomycin, (6) penicillin, and (7) bacitracin. The curves for polymyxin and aerosporin are parallel and close to each other and the same is true for chloromycetin and aureomycin. The

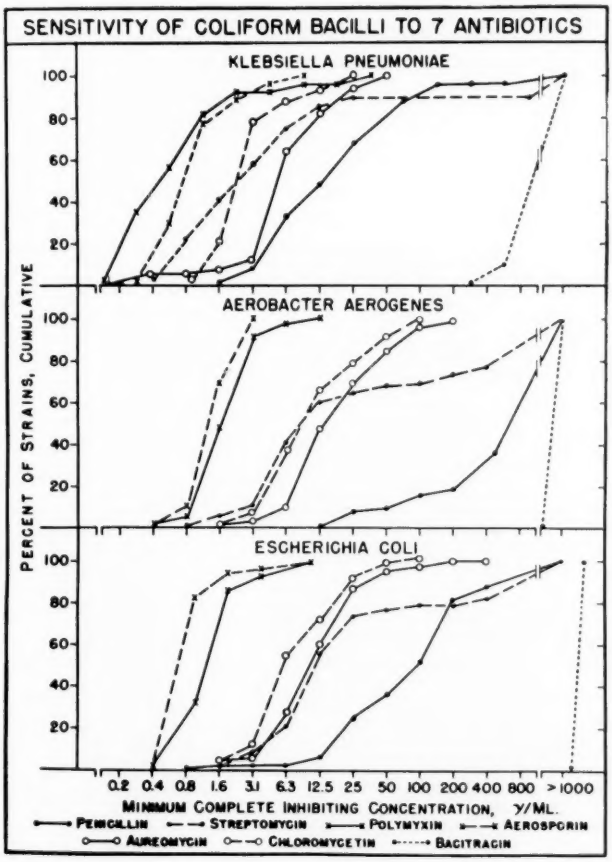

Fig. 2.

streptomycin curve is similar and falls close to the latter two curves for a height equivalent to about two-thirds of the strains; the remainder of the streptomycin curve is flatter, indicating the occurrence of more strains of increasing resistance to streptomycin and corresponding to the late rise noted in Fig. 1. The curve for penicillin falls far over to the right and its slope is a slow one because of the relatively small proportion of sensitive strains.

Aerobacter aerogenes.—The results of tests for sensitivity of twenty-three recently isolated pathogenic strains of *A. aerogenes* simultaneously carried out by the plate dilution method with all seven antibiotics are shown in Table IV. These results, on the whole, are quite similar to those obtained with *Esch. coli*

TABLE IV. SENSITIVITY OF TWENTY-THREE STRAINS OF A. AEROGENES TO SEVEN ANTIBIOTICS

STRAIN	SOURCE	PENICILLIN		STREPTOMYCIN		BACITRACIN		POLYMYXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
		C	P	C	P	C	P	C	P	C	P	C	P	C	P
1	Urine	6,000	3,000	3.1	1.6	10,000	5,000	6.3	1.6	3.1	1.6	6.3	3.1	3.1	--
2	Blood	6,000	--	6.3	3.1	10,000	--	1.6	--	1.6	--	12.5	3.1	3.1	--
3	Urine	6,000	--	6.3	3.1	10,000	--	1.6	--	1.6	--	12.5	--	6.3	3.1
4	Blood	1,500	--	6.3	3.1	10,000	--	3.1	1.6	3.1	0.8	12.5	--	6.3	3.1
5	Blood	375	188	6.3	3.1	10,000	--	1.6	--	3.1	0.8	12.5	6.3	3.1	1.6
6	Wound	> 6,000	6,000	> 1,000	--	> 10,000	10,000	1.6	--	3.1	0.8	12.5	6.3	6.3	3.1
7	Urine	> 6,000	--	> 1,000	--	> 10,000	10,000	3.1	1.6	3.1	0.8	12.5	6.3	12.5	6.3
8	Urine	> 6,000	6,000	> 1,000	--	> 10,000	--	3.1	1.6	1.6	--	12.5	6.3	50.0	3.1
9	Sputum	6,000	--	6.3	3.1	10,000	5,000	3.1	1.6	1.6	--	12.5	6.3	12.5	6.3
10	Urine	750	--	6.3	3.1	10,000	--	1.6	--	1.6	--	12.5	6.3	6.3	3.1
11	Urine	6,000	3,000	25.0	6.3	> 10,000	10,000	1.6	--	1.6	--	12.5	3.1	6.3	3.1
12	Urine	> 6,000	--	6.3	3.1	10,000	5,000	1.6	--	1.6	--	12.5	6.3	50.0	3.1
13	Urine	750	375	6.3	3.1	> 10,000	10,000	3.1	1.6	1.6	--	100.0	50.0	50.0	--
14	Urine	1,500	--	6.3	3.1	> 10,000	10,000	0.8	--	1.6	0.8	12.5	--	12.5	3.1
15	Urine	1,500	--	> 1,000	--	10,000	--	25.0	1.6	3.1	0.8	12.5	6.3	50.0	3.1
16	Blood	750	375	6.3	3.1	10,000	--	1.6	--	1.6	0.8	12.5	6.3	12.5	3.1
17	Urine	> 6,000	--	6.3	3.1	10,000	--	1.6	--	3.1	0.8	12.5	6.3	6.3	--
18	Urine	750	375	6.3	3.1	10,000	--	1.6	--	1.6	--	25.0	6.3	12.5	6.3
19	Urine	> 6,000	--	12.5	3.1	10,000	--	3.1	1.6	1.6	--	12.5	6.3	12.5	3.1
20	Urine	> 6,000	3,000	6.3	3.1	10,000	5,000	3.1	1.6	1.6	--	12.5	6.3	25.0	3.1
21	Urine	6,000	3,000	> 1,000	--	10,000	5,000	3.1	--	1.6	--	12.5	6.3	6.3	--
22	Urine	3,000	--	3.1	1.6	5,000	2,500	3.1	1.6	0.8	--	12.5	6.3	6.3	3.1
23	Urine	1,500	750	0.8	0.4	5,000	2,500	3.1	1.6	0.8	--	200.0	50.0	50.0	25.0
98*	Stock	0.02	0.01	N.D.	--	0.9	0.5	N.D.	--	N.D.	--	1.6	0.8	N.D.	--
T†	Stock	N.D.	--	1.6	0.8	N.D.	--	N.D.	--	N.D.	--	N.D.	--	3.1	1.6
McL‡	Stock	N.D.	--	N.D.	--	N.D.	--	1.6	0.8	1.6	0.8	N.D.	--	N.D.	--

All sensitivities expressed in γ per milliliter.
C, complete inhibition; P, partial inhibition.
—, Full growth in next dilution; N.D., not done.
*Reference culture, Streptococcus 98.
†Reference culture, *K. pneumoniae*, Type A.
‡Reference culture, *Esch. coli*, McLeod strain.

and shown in Table II. There were some obvious quantitative differences, however, which were probably significant. For each of the antibiotics there were more strains of *A. aerogenes* that required high concentration to produce inhibition than were noted in the case of the strains of *Esch. coli.* This is particularly evident with respect to penicillin. In the case of streptomycin, however, although there was a greater proportion of highly resistant strains of *A. aerogenes,* the majority were inhibited by slightly lower concentrations than those required for most of the strains of *Esch. coli.* All of the other observations that were made with respect to the strains of *Esch. coli* are equally apparent for these strains of *A. aerogenes.*

The data for all the strains of *A. aerogenes* that were tested are shown in Table V and the curves for the cumulative distribution of the results of the sensitivity tests with all of the antibiotics are shown in the middle section of Fig. 2. The similarities of these results and those obtained with the strains of *Esch. coli* are more readily apparent from a comparison of the curves in the lower two sections of Fig. 2. Only the curves for streptomycin and penicillin show any significant quantitative differences although the general shape of the curves for the two organisms obtained with each of these antibiotics is quite similar. The differences correspond to those observed for the smaller groups of strains listed in Tables II and IV and have already been noted. The curve representing the penicillin sensitivities of *A. aerogenes* starts further to the right, rises much more slowly at first and then more rapidly in the high range of concentrations, reflecting the much greater penicillin resistance of *A. aerogenes* as compared with *Esch. coli.*

The order of the in vitro effectiveness of the different antibiotics against the strains of *A. aerogenes,* as seen from Table V and Fig. 2, is quite similar to the order of their effectiveness against the strains of *Esch. coli* except in the case of streptomycin. About two-thirds of the strains of *A. aerogenes* appear to be as sensitive to streptomycin as they are to chloromycetin or aureomycin, while the remaining strains are much more resistant to streptomycin.

Klebsiella pneumoniae.—The results of the tests for sensitivity of twenty-three recently isolated strains of *K. pneumoniae* carried out by the plate dilution method simultaneously with seven antibiotics are shown in Table VI. Again these results are qualitatively quite similar to those obtained with the other coliform organisms already presented. The outstanding quantitative differences are seen in the tests with penicillin and streptomycin. These indicate that the Klebsiellae were, on the whole, much more sensitive to both of these antibiotics than were the strains of *Esch. coli* and *A. aerogenes.* There is also some indication that the strains derived from sputum and blood which were predominantly Group A strains were the most sensitive to these two antibiotics.

It is also of interest to note in passing that two of the strains (Strains 2 and 11) were among the most resistant to all of the antibiotics and a third (Strain 12) was among the least sensitive to all antibiotics except streptomycin. With the other strains, as with those of *Esch. coli* and *A. aerogenes,* there appeared to be no relation between the sensitivity of any strain to any one antibiotic and its sensitivity or resistance to any of the others.

TABLE V. SENSITIVITY OF PATHOGENIC STRAINS OF A. AEROGENES TO SEVEN ANTIBIOTICS

MINIMUM INHIBITING CONCENTRATION* (γ/ML.)	PENICILLIN		STREPTOMYCIN		BACITRACIN		POLYMIXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL
0.8			1	0.5			1	4	2	9				
1.6			6	3.1			10	44	14	61				
3.1			15	7.7			10	44	7	30	2	1.1	3	8
6.3			64	32.7			1	4			15	8.2	13	34
12.5			31	15.8			0				70	38.0	9	24
25	2	6	14	7.1			1	4			44	23.9	5	13
50	1	3	4	2.0							27	14.7	5	13
100	2	6	4	2.0							22	12.0	3	8
200	1	3	6	3.1							4	2.1		
400-750	2	6	5	2.6										
> 1,000	26	76	46	23.5	23	100								
Total	34	100	196	100	23	100	23	100	23	100	184	100	38	100

*Complete inhibition.

TABLE VI. SENSITIVITY OF TWENTY-THREE STRAINS OF K. PNEUMONIAE TO SEVEN ANTIBIOTICS

STRAIN	TYPE	SOURCE	PENICILLIN		STREPTOMYCIN		BACITRACIN		POLYMYXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
			C	P	C	P	C	P	C	P	C	P	C	P	C	P
1	A	Sputum	20	10	1.6	—	5,000	—	1.3	0.6	1.3	0.6	12.5	3.1	3.1	1.6
2	X	Urine	600	150	6.3	3.1	20,000	10,000	2.5	1.3	1.3	—	50.0	25.0	25.0	6.3
3	X	Urine	300	150	6.3	3.1	10,000	—	1.3	0.6	5.0	0.6	12.5	—	6.3	1.6
4	A	Blood	10	5	1.6	—	2,500	—	1.3	0.6	0.6	—	6.3	—	3.1	0.8
5	A	Spinal fluid	10	5	0.8	—	2,500	1,250	0.6	0.3	0.6	—	6.3	3.1	3.1	0.4
6	A	Blood	10	5	0.8	—	2,500	1,250	0.3	—	1.3	0.6	6.3	3.1	3.1	0.4
7	A	Urine	5	—	0.8	—	2,500	1,250	0.3	—	1.3	0.6	6.3	3.1	1.6	0.8
8	A	Sputum	5	—	0.8	—	2,500	1,250	0.6	—	1.3	0.6	6.3	3.1	3.1	0.8
9	C	Sputum	10	—	3.1	0.8	2,500	—	1.3	0.3	1.3	0.6	6.3	—	1.6	0.8
10	A	Abscess	5	—	0.8	—	2,500	—	0.3	—	1.3	0.6	6.3	—	3.1	0.8
11	C	Urine	>600	—	>1,000	—	>10,000	—	2.5	1.3	2.5	0.6	25.0	—	25.0	3.1
12	B	Urine	300	150	3.1	1.6	10,000	—	10.0	1.3	10.0	0.6	25.0	12.5	12.5	3.1
13	C	Sputum	150	—	3.1	1.6	2,500	—	1.3	0.6	0.6	—	6.3	—	3.1	1.6
14	A	Sputum	5	—	1.6	0.8	5,000	—	1.3	0.6	0.6	—	6.3	3.1	3.1	1.6
15	A	Sputum	10	—	3.1	1.6	5,000	—	0.6	0.3	1.3	0.6	6.3	3.1	3.1	1.6
16	A	Sputum	10	5	0.8	0.4	2,500	1,250	0.3	—	1.3	0.6	6.3	3.1	1.6	0.8
17	A	Blood	10	5	1.6	0.8	1,250	625	0.3	—	1.3	0.6	6.3	3.1	3.1	1.6
18	X	Sputum	150	—	3.1	1.6	5,000	—	40.0	0.3	5.0	0.6	12.5	6.3	6.3	3.1
19	A	Sputum	75	10	0.8	—	625	—	0.3	0.2	1.3	0.6	12.5	3.1	1.6	0.8
20	A	Sputum	5	—	0.8	—	2,500	—	0.6	0.3	1.3	0.6	12.5	3.1	3.1	0.8
21	D	Sputum	20	—	0.8	0.4	2,500	—	0.3	0.2	0.6	0.3	12.5	6.3	1.6	0.3
22	A	Sputum	5	—	0.8	0.4	625	—	0.3	0.2	0.6	—	6.3	3.1	3.1	0.8
23	A	Sputum	20	10	1.6	0.8	2,500	1,250	0.6	0.3	0.6	—	6.3	—	3.1	1.6
98*		Stock	0.005	—	N.D.	N.D.	0.9	0.5	N.D.	N.D.	N.D.	N.D.	1.6	0.8	N.D.	N.D.
T†	A	Sputum	N.D.	N.D.	1.6	0.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	6.3	1.6	3.1	1.6
McL‡		Stock	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	0.6	0.2	2.5	0.6	N.D.	N.D.	N.D.	N.D.

Sensitivities expressed in γ per milliliter.

C, complete inhibition; P, partial inhibition (few colonies or many tiny colonies).

—, Full growth in next dilution; N.D., not done.

*Reference strain, Streptococcus 98.

†Reference strain. *K. pneumoniae.* Type A, strain T.

‡Reference strain. *Esch. coli,* McLeod strain.

TABLE VII. SENSITIVITY OF STRAINS OF K. PNEUMONIAE TO SEVEN ANTIBIOTICS

COMPLETE INHIBITION (γ/ML.)	PENICILLIN		STREPTOMYCIN		BACITRACIN		POLYMYXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL
0.2-0.4			3	2.9			8	35			1	3		
0.6-0.8			21	20.6			5	22	7	30	0			
1.3-1.6			19	18.6			6	26	12	52	1	3	5	22
2.5-3.1	3	7	17	16.7			2	9	1	4	1	3	13	56
5-6.3	12	26	17	16.7			0		2	9	21	55	2	9
10-12.5	7	15	10	9.8			1	4	1	4	7	18	1	4
20-25.	9	20	4	3.9			0				6	16	2	9
40-50	0		2	2.0			1	4			1	3		
75-150	9	20												
300	4	9												
625	0				2	9								
>1,000	2	4	9	8.8	21	91								
Total	46	100	102	100	23	100	23	100	23	100	38	100	23	100

The findings for all of the strains are summarized in Table VI, and the cumulative distribution curves are shown in the upper section of Fig. 2. The greater sensitivity of the strains of Klebsiellae as a whole to all of the antibiotics is at once apparent. In Fig. 2, this is shown by the fact that the curves for the Klebsiellae fall further to the left as compared with those for the strains of *Esch. coli* and *A. aerogenes* as shown in the lower sections of the same figure. Particularly noteworthy in this respect are the curves for streptomycin and penicillin.

An analysis of the data was made with respect to the sources of all the strains of Klebsiella that were tested for streptomycin, of which there was a greater number than those tested with other antibiotics. This showed that the great majority of strains obtained from sputum and blood, most of which were Type A strains, were inhibited by streptomycin concentrations of 3.1 γ per milliliter or less, whereas those obtained from urine, most of which could not be classified serologically, required 6.3 γ per milliliter or more for complete inhibition.

The order of the in vitro effectiveness of the strains of *K. pneumoniae* to these antibiotics, on a weight basis, is as follows: polymyxin, aerosporin, chloromycetin, aureomycin, penicillin, and bacitracin, with streptomycin occupying an anomalous position. About half of the strains were slightly more sensitive to streptomycin than to chloromycetin or aureomycin; about 10 per cent of the strains were more resistant to streptomycin than to either chloromycetin or aureomycin, and the remaining strains were inhibited by concentrations of streptomycin similar to those required for chloromycetin and aureomycin to inhibit the majority of the strains.

DISCUSSION

The findings that have been presented should be of interest because they offer a useful and fairly reliable comparison of a group of antibiotics with divergent properties against a group of organisms which appears to be assuming considerable clinical importance. They are of particular value because of the uniform methods by which the data have been obtained and because the strains studied were recently isolated from clinical sources and from infections in which antibiotic therapy was being used or contemplated.

The tables and the charts depict most of the natural variations in the activity of the antibiotic agents studied against the three species of coliform organisms. By way of summary and in order to simplify the application of these data, it may be of interest to compare, on a weight basis, the range of concentrations of each of the antibiotics that would be required to inhibit the majority of the strains of each of these species. Taking two-thirds of the strains as a minimum proportion for this purpose, these values as derived from the data presented are shown in Table VIII.

On the whole, polymyxin and aerosporin were found to be very similar to each other in almost every respect and were the most active agents in this group. Likewise, aureomycin and chloromycetin were remarkably similar in their action

against each of these organisms; they were appreciably less active than polymyxin and aerosporin but much more active than penicillin and bacitracin. Streptomycin occupies a sort of dual position in that for about two-thirds of the strains its action was quantitatively similar to that of aureomycin and chloromycetin but the remaining strains required larger concentrations of streptomycin and a significant proportion of the strains could be considered as resistant to the latter agent. Bacitracin, from a practical point of view, may be considered to be without effective on any of these coliform organisms. Penicillin, on the other hand, though much less active than aureomycin and cholormycetin, was nevertheless quite active against *K. pneumoniae* and only slightly less so against a considerable proportion of the strains of *Esch. coli*, but it was essentially inactive against *A. aerogenes*.

From the point of view of the different coliform organisms, the strains of *K. pneumoniae* were generally the most sensitive to all of the antibiotics, the strains of *A. aerogenes* were the most resistant of this group, and those of *Esch. coli* had an intermediate position.

TABLE VIII. RANGE OF CONCENTRATIONS WHICH COMPLETELY INHIBITS TWO-THIRDS OR MORE OF THE STRAINS

	ESCH. COLI		A. AEROGENES		K. PNEUMONIAE	
ANTIBIOTIC	M.I.C. (γ/ML.)	% OF STRAINS	M.I.C. (γ/ML.)	% OF STRAINS	M.I.C. (γ/ML.)	% OF STRAINS
Penicillin	25-200	79	>1,000	76	5-75	78
Streptomycin*	6.3-25	67	1.6-25	67	0.8-6.3	73
Bacitracin	5,000-10,000	100	5,000-10,000	74	2,500-5,000	70
Polymyxin	1-2	85	1.6-3.1	88	0.3-1.3	83
Aerosporin	1-2	96	1.6-3.1	91	0.6-1.3	82
Aureomycin	6.3-25	82	12.5-50	77	6.3-25	89
Chloromycetin	6.3-25	85	6.3-50	84	1.6-3.1	78

M.I.C., minimum (complete) inhibiting concentrations.

*Resistant strains (requiring >1,000 γ/ml.): *Esch. coli*, 16 per cent; *A. aerogenes*, 24 per cent; *K. pneumoniae*, 9 per cent.

From the point of view of the biology of this group of organisms, the distinctive patterns of the distribution of the sensitivities of each of the species of organisms to the various antibiotics are of interest. This has been previously observed in the case of two species of Neisseria, namely, the gonococcus and the meningococcus, with respect to their sensitivity to sulfonamides and penicillin. In that particular instance, these relationships have proved to be the determining factors in the choice of therapy. The sulfonamides are by far the most effective agents against meningococcal infections and have been all but abandoned as a therapy of gonorrhea. On the other hand, penicillin has proved to be the treatment of choice for gonorrhea and is much less effective in meningococcal infections. In the present instance, the generally greater resistance of strains of *A. aerogenes* and the greater sensitivity of the Klebsiella strains to most of the antibiotics is of similar interest and seems to be reflected in the results of antibiotic therapy of infections with these strains.*

*The possibility that certain organisms contain or secrete enzymes capable of specifically inactivating one or another of the antibiotics must also be considered. To our knowledge, the only authentic examples are the production of penicillinase by certain coliform and other organisms[2-4] and the enzyme which hydrolyzes chloromycetin.[5]

As to the clinical significance of the quantitative data that have been presented, it is essential to bear in mind the manner in which these data were derived and the limitations of direct application of such in vitro data to actual therapy. Mention of some of these limitations was made in the introductory paragraphs. It is also worth noting that the conditions of the tests, as carried out in this study, tend to favor some antibiotics and to be unfavorable to others. Chloromycetin, for example, is quite stable under these conditions; streptomycin is more active at the alkaline pH of these tests, while aureomycin loses activity rapidly under the same conditions. It should be emphasized further that the actual values that are given here are strictly valid only for the exact conditions of these tests and within the rather wide limits of error of such biologic methods. Quantitative comparisons with similar data in which other materials and methods are used must, therefore, be made with these reservations.

Because of the manner in which the end points have been chosen, the values for the sensitivities in the present study are much higher than those given by many other workers. On the whole, even the figures for "partial inhibition," as defined in this study, may be many times greater than values for sensitivity that are based on "50 per cent inhibition" end points. The differences are greater still if the end point is chosen as the dilution which produces the least detectable reduction in the amount of growth as compared with controls in similar media that contains no antibiotic and they are further exaggerated if electrophotometric methods are used for comparisons of densities of growth.

This is not the place to review the factors that are known to influence antibiotic activity during actual therapy of infections. The importance of alkalinization in the success of streptomycin therapy of urinary tract infection may serve as an example of one such factor that is obviously applicable to that antibiotic but has not been shown to be of importance in the case of other antibiotics. Furthermore, time-dose relationships depend upon the absorption, excretion, and distribution of the agents in the body, which in turn determine the concentration of the antibiotic at the site where the organisms are multiplying. The nature of the lesion, the conditions that prevail at the site of infection, and the accessibility of the antibiotic to the bacteria are other determining factors. All of these factors may vary with the offending organism or with the antibiotic.

Furthermore, it goes almost without saying that the toxicity of one agent or the innocuous nature of another may sometimes be the prime factor that influences the choice between effective antibiotics. Thus, both polymyxin and aerosporin, which, from the results of all the in vitro data that have been presented, are by far the agents of choice against all of the coliform organisms that were studied, have failed to achieve more than the most limited clinical trials because of the readiness with which they produce serious renal damage. Whether or not either of these agents will prove useful for topical therapy or for use in the intestinal tract must, of necessity, depend on the extent to which they are absorbed from the sites of application.

It is hoped that, in spite of all these reservations, the data that have been presented will prove useful to those who are concerned with the clinical and laboratory control of antibiotic therapy.

SUMMARY AND CONCLUSIONS

The results of tests for sensitivity to seven antibiotics carried out simultaneously on a number of recently isolated, pathogenic strains of *Esch. coli* have been presented. They serve to indicate the variations in the in vitro activity of these antibiotics with different strains of the same species. The results of similar tests with strains of *A. aerogenes* and *K. pneumoniae* also have been presented.

These data together with the results of all other tests for sensitivity to these antibiotics that have been carried out in this laboratory with strains of these organisms have been analyzed and compared.

Under the conditions of these tests, the most effective antibiotics against all of these organisms were polymyxin and aerosporin and both of these agents were about equally active. Aureomycin and chloromycetin were about equally effective against each of the species and both were appreciably less active than polymyxin and aerosporin. Streptomycin ranked with aureomycin and chloromycetin in its activity against a large proportion of the strains, but there was an appreciable number of strains of each of these organisms that was highly resistant to streptomycin. Penicillin showed the greatest variation in its in vitro effect on the different species of coliform bacilli; it was moderately effective against *K. pneumoniae,* less so against most strains of *Esch. coli,* and essentially inactive against *A. aerogenes.*

On the whole, the strains of *K. pneumoniae* were the most sensitive, those of *A. aerogenes* were the most resistant, while the strains of *Esch. coli* were intermediate in their susceptibility to all seven antibiotics.

The limitations of these in vitro data with respect to their clinical application have been discussed.

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IN VITRO SENSITIVITY OF BACILLUS PROTEUS AND PSEUDOMONAS AERUGINOSA TO SEVEN ANTIBIOTICS (PENICILLIN, STREPTOMYCIN, BACITRACIN, POLYMYXIN, AEROSPORIN, AUREOMYCIN, AND CHLOROMYCETIN)

PAUL F. FRANK, M.S., CLARE WILCOX, AND MAXWELL FINLAND, M.D.
BOSTON, MASS.

INFECTIONS with proteus and pyocyaneus organisms have assumed a new importance since the introduction and extensive use of therapeutically potent chemical and antibiotic agents. Infections with these organisms seem to be highly resistant to treatment with the antimicrobial agents now available and frequently they either persist or replace infections with other organisms after such therapy is used. This is particularly true in chronic urinary tract infections, in chronic bronchopulmonary suppurations, and in infected wounds and burns. Since great reliance is placed upon the use of antibiotics in the management of such suppurative processes and since it has become a frequent if not a universal practice to exhaust the possibilities of the available antibiotics in these conditions, it is well to have some understanding of the limitations of such therapy.

The first essential for successful therapy with antibacterial agents is the susceptibility of the infecting organisms to these agents. In the present paper are presented the results of in vitro tests for sensitivity to seven antibiotics of a number of recently isolated pathogenic strains of *Bacillus proteus* and *Pseudomonas aeruginosa*. These tests were carried out in a manner similar to those reported for various coliform organisms.[1] A group of strains of each organism was tested simultaneously with all seven antibiotics in order to indicate some of the variations in the relative sensitivity of individual strains to the different antibiotics. Similar tests were carried out with other strains in the course of the clinical study and control of infections in which treatment with some of these antibiotics was undertaken or contemplated. All of these tests were combined and analyzed. In this manner it was possible to obtain a more reliable picture of the effectiveness of each antibiotic against different strains and to compare the potency of the different antibiotics against each of these organisms.

MATERIALS AND METHODS

Sources of the Strains.—The great majority of the strains were obtained from cultures of infected urine, but a significant proportion were obtained from the sputum of patients with bronchopulmonary infections and from infected wounds and exudates. The sources of the strains are listed in Table I. Most of these materials were obtained from the patients before antibiotic therapy was started, but some of the patients had received antibiotics for the same or other infections on previous occasions.

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital and the Department of Medicine, Harvard Medical School.
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TABLE I. SOURCES OF THE STRAINS STUDIED

SOURCE	B. PROTEUS		PS. AERUGINOSA	
	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL
Urine	178	74.5	160	59.9
Sputum	21	8.8	32	12.0
Exudate	21	8.8	51	19.1
Wound	13	5.4	19	7.1
Blood	1	0.4	1	0.4
Feces	5	2.1	4	1.5
Total	239	100	267	100

Sensitivity Tests.—The antibiotics used in this study,* the manner in which they were prepared for the tests, the culture media used, and the methods for carrying out and interpreting the tests for sensitivity were the same as those used in the study of the coliform organisms.[1] When proteus strains were tested by the plate dilution method, a maximum of four strains were streaked on each agar plate and these plates were examined at frequent intervals during the first twenty-four hours in order to observe any swarming tendency on the part of some uninhibited strains which might obscure the growth or inhibition of strains in adjacent segments. In the dilutions of antibiotic near the end points, however, there was no tendency to swarm and no difficulty was encountered on that score.

RESULTS

The data concerning the proteus and pyocyaneus organisms will be presented separately. In each instance the results of the tests that were carried out simultaneously with several strains and all of the antibiotics will be presented first and the findings with respect to all of the tests including those carried out with individual antibiotics as they were used clinically will then be analyzed.

Bacillus proteus.†—The results of tests for sensitivity of eighteen strains of *B. proteus* carried out by the plate dilution method with all seven antibiotics are listed in Table II. As with other organisms, the general lack of relationship between the sensitivity of individual strains to the different antibiotics is apparent. The same is true, in general, of the variations in the sensitivity of different strains to the same antibiotic.

The end points for both "complete" and "partial" inhibitions are shown in Table II. The "range of partial inhibition," that is, the difference between the concentrations of antibiotic required for complete and partial inhibition as defined in these studies,[1] varied from strain to strain and also among the different antibiotics. Sharper end points, however, were more frequently observed in the tests carried out by the tube dilution method that was generally used in the "clinical" sensitivity tests. Thus, among the streptomycin sensitivity tests carried out by the latter method, over 40 per cent of the strains yielded a sharp end point (i.e., essentially full growth in the dilution beyond the one showing complete inhibition) and the spread between the end points for complete and partial inhibition was only a single twofold dilution for two-thirds of the re-

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†The great majority of the strains were classified as *Proteus vulgaris,* but a few strains of *P. morganii* are included.

TABLE II. SENSITIVITY OF EIGHTEEN STRAINS OF B. PROTEUS TO SEVEN ANTIBIOTICS

STRAIN	SOURCE	PENICILLIN		STREPTOMYCIN		BACITRACIN		POLYMYXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
		C	P	C	P	C	P	C	P	C	P	C	P	C	P
1	Wound	20	10	50	12.5	>10,000	10,000			>200	—	400	200	3.1	—
2	Wound	40	20	50	12.5	>10,000	—			>200	—	400	200	50.0	6.3
3	Urine	40	20	50	12.5	>10,000	—			>200	—	400	200	25.0	6.3
4	Wound	10	—	50	12.5	10,000	5,000			>200	—	400	100	25.0	6.3
5	Urine	10	—	50	12.5	10,000	—			>200	—	200	50	12.5	6.3
6	Urine	20	10	50	12.5	10,000	—	All strains		>200	—	25	—	12.5	6.3
7	Urine	20	10	50	12.5	>10,000	—			>200	—	400	200	25.0	6.3
8	Ear	10	—	50	12.5	10,000	5,000	grew pro-		>200	—	200	100	25.0	6.3
9	Urine	>1,200	—	>1,000	—	10,000	5,000			200	100	50	—	12.5	6.3
10	Urine	10	—	50	12.5	10,000	—	fusely in		>200	—	200	—	12.5	—
11	Urine	20	10	50	12.5	>10,000	10,000			>200	—	400	200	3.1	—
12	Urine	>1,200	—	>1,000	—	>10,000	—	200 γ/ml.		>200	—	25	12.5	25.0	12.5
13	Urine	>1,200	—	25	6.3	>10,000	10,000			>200	—	25	12.5	50.0	—
14	Urine	20	10	50	12.5	10,000	5,000			>200	—	200	100	6.3	—
15	Urine	20	10	25	6.3	10,000	5,000			>200	—	200	100	25.0	3.1
16	Ear	40	—	50	25.0	>10,000	10,000			>200	—	400	200	12.5	—
17	Eye	300	75	12.5	6.3	10,000	5,000			200	—	12.5	—	6.3	—
18	Urine	>1,200	—	>1,000	—	10,000	5,000			>200	—	400	200	25.0	3.1
98*	Stock	0.02	0.01	N.D.	N.D.	0.45	0.23	N.D.	N.D.	N.D.	N.D.	0.8	0.4	3.1	1.6
T†	Stock	N.D.	N.D.	1.6	0.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
McL‡	Stock	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.25	0.63	1.25	0.63	N.D.	N.D.	N.D.	N.D.

Sensitivities expressed in γ per milliliter.
C, complete inhibition; P, partial inhibition (few colonies or many tiny colonies).
—, Full growth in next dilution; N.D., not done.
*Reference culture, Streptococcus, strain 98.
†Reference culture, Type A Friedländer's bacillus, strain T.
‡Reference culture, *Esch. coli*, McLeod strain.

maining strains. In the case of the aureomycin sensitivities of the proteus strains, 60 per cent of the strains gave a sharp end point and the end point for partial inhibition was only rarely found to be more than a single twofold dilution beyond that required for complete inhibition.

Of considerable interest is the relative sensitive of the majority of the strains to penicillin, being exceeded for most strains only by their sensitivity to chloromycetin. Of the four strains among those listed in Table II that were highly resistant to penicillin, at least two (strains 12 and 13) were obtained from patients who were known to have received both penicillin and streptomycin on

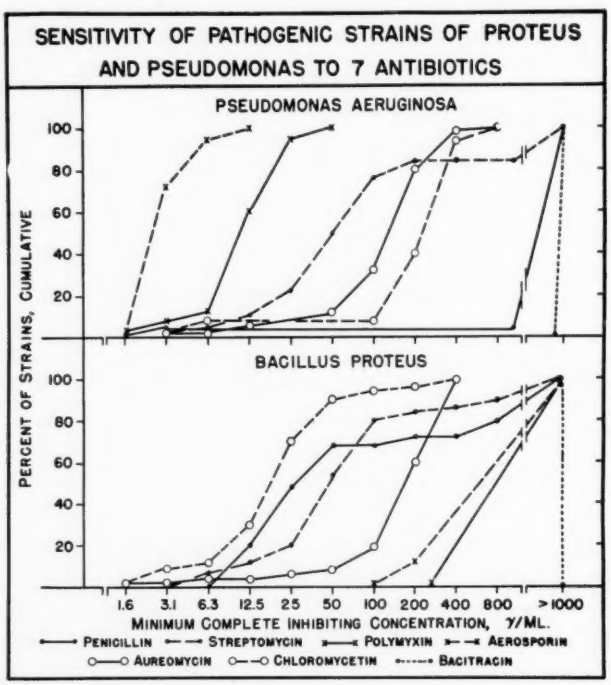

Fig. 1.

previous occasions although they were not being treated with any antibiotics at the time these organisms were isolated. It is also possible that the patients from whom Strains 9 and 18 were isolated also had received these antibiotics in the past, although this was denied by the patients and there was no record of such therapy.

It should also be noted that three of these penicillin-resistant strains were also highly resistant to streptomycin. The fourth, however, was relatively sensitive to the latter agent although it was obtained from one of the patients who had previously been treated with streptomycin.

With respect to bacitracin, these proteus strains were all highly resistant and resembled the various coliform bacilli in this respect. The rather unexpected finding was the resistance of all of these proteus strains to both polymyxin

and aerosporin. This was in sharp contrast to the various coliform bacilli, all of which were more sensitive to polymyxin and aerosporin than to any of the other five antibiotics. Aureomycin, too, was relatively ineffective against most, though not all, of these strains. Furthermore, most of these strains of *B. proteus* were considerably more sensitive to chloromycetin than to aureomycin, though occasional ones were equally sensitive to both agents. Such a marked difference between the in vitro effects of these two agents was not noted in the case of any of the other pathogenic bacteria that were studied.

The results of the tests for sensitivity of all of the pathogenic strains of *B. proteus* that were assayed, including those shown in Table II, are summarized in Table III. Only the complete inhibiting concentrations are given in this table and the curves representing the cumulative per cent of strains with increasing resistance to each of the antibiotics are shown in the lower half of Fig. 1. These curves and the data in Table III upon which they are based show clearly the relative in vitro effectiveness of each of the antibiotics against all of the strains that were assayed. The most effective of these seven antibiotics, as shown from the position of the curve farthest to the left, was chloromycetin. Penicillin, streptomycin, and aureomycin followed in that order, but a significant proportion of the strains were moderately to highly resistant to streptomycin and even more were resistant to penicillin. Far over to the right are the curves for aerosporin, polymyxin, and bacitracin, indicating the resistance of most or all of the strains to these agents.

Pseudomonas aeruginosa.—The results of tests for sensitivity to the various antibiotics carried out by the plate dilution method with twenty-five recently isolated, pathogenic strains of pyocyaneus organisms are shown in Table IV.* It is at once apparent that the activity of some of the antibiotics against these strains was markedly different from their activity against the proteus strains shown in Table II. This was true of penicillin, polymyxin, aerosporin, and chloromycetin. Penicillin was inactive against all of the strains listed in Table IV, though it was quite effective against most of the proteus strains. The reverse was true of aerosporin and polymyxin; they were inactive against the proteus strains and were quite effective against these pseudomonas organisms. Chloromycetin was much less active against the latter strains than it was against the proteus strains. On the other hand, streptomycin and aureomycin differed only slightly in their action against the different organisms, while bacitracin was inactive against both the proteus and the pyocyaneus strains.

The end points for both complete and partial inhibition are shown in Table IV. On the whole, the end points were quite sharp with these strains even in those tests which were carried out by the plate dilution method. The same was true of the strains tested for clinical purposes by the tube dilution method; only rarely was the difference between the end points for complete and partial inhibition more than a single twofold dilution.

*The tests with all twenty-five of these strains were carried out simultaneously with only six antibiotics. The chloromycetin was not made available to us until after these tests were completed and by that time some of the strains were lost.

TABLE III. SENSITIVITY OF PATHOGENIC STRAINS OF B. PROTEUS TO SEVEN ANTIBIOTICS

M.I.C. (γ/ML.)	PENICILLIN		STREPTOMYCIN		BACITRACIN		POLYMYXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL
3.1											2	1.8	3	6
6.3			4	3.5							0		2	4
10-12.5	5	20	10	8.8							1	0.9	11	20
20-25	7	28	10	8.8							3	2.7	21	39
40-50	5	20	42	36.8							3	2.7	11	20
100	0		25	21.9							12	10.7	2	4
200-300	1	4	8	7.0					2	11	47	42.0	2	4
400-500	0		2	1.8					0		44	39.3	2	4
750-1,000	2	8	2	1.8					0					
>1,000	5	20	11	9.6	18	100	18*	100	16*	89				
Total	25	100	114	100	18	100	18	100	18	100	112	100	54	100

M.I.C., minimum concentration for complete inhibition.
*>200 γ per milliliter, the highest concentration used.

TABLE IV. SENSITIVITY OF TWENTY-FIVE STRAINS OF PS. AERUGINOSA TO SEVEN ANTIBIOTICS

STRAIN	SOURCE	PENICILLIN	STREPTOMYCIN		BACITRACIN	POLYMYXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
		C	C	P	C	C	P	C	P	C	P	C	P
1	Urine		100	50.0		12.5	—	2.5	1.3	200	—	200	25
2	Urine		100	50.0		12.5	—	5.0	2.5	200	—	200	50
3	Abscess		500	—		6.3	—	2.5	1.3	200	100	400	100
4	Urine		1,000	—		25.0	12.5	2.5	—	200	100	1,000	
5	Abscess		1,000	—		6.3	—	2.5	1.3	200	100	200	100
6	Sputum		1,000	—		12.5	—	5.0	2.5	100	—	400	100
7	Ulcer		50	25.0		12.5	—	5.0	2.5	200	100	N.D.	
8	Urine		200	50.0		12.5	—	2.5	—	1,000	—	N.D.	
9	Urine	All strains	1,000	—		12.5	—	2.5	—	200	—	200	100
10	Urine		100	50.0	All strains	25.0	12.5	2.5	—	200	—	400	50
11	Urine	grew pro-	1,000	—		12.5	—	2.5	—	100	50	200	50
12	Ear		50	—	grew pro-	12.5	—	2.5	—	100	50	N.D.	
13	Pleura	fusely in	100	50.0		12.5	—	2.5	—	200	—	400	100
14	Ear		100	50.0	fusely in	50.0	12.5	2.5	—	100	—	200	100
15	Ear	1,500	100	25.0		25.0	12.5	2.5	—	200	100	400	100
16	Urine		50	12.5	10,000 γ/ml.	3.1	—	2.5	—	50	—	400	200
17	Abscess	γ/ml.	200	50.0		12.5	—	2.5	—	200	100	N.D.	
18	Burn		100	—		25.0	12.5	2.5	—	100	—	200	100
19	Ear		100	—		25.0	—	10.0	2.5	200	100	N.D.	
20	Urine		100	50.0		12.5	—	2.5	—	200	100	400	100
21	Abscess		200	50.0		25.0	12.5	2.5	—	200	100	400	100
22	Wound		100	50.0		25.0	12.5	2.5	—	200	100	N.D.	
23	Ear		100	—		25.0	12.5	5.0	2.5	400	—	N.D.	
24	Urine		100	50.0		25.0	—	5.0	2.5	200	100	400	200
25	Sputum		100	50.0		12.5	—	5.0	2.5	100	—	N.D.	
98*	Stock	0.02	N.D.	N.D.	0.9	N.D.	N.D.	N.D.	N.D.	1.6	0.8	3.1	—
T†	Stock	N.D.	1.6	0.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
McL‡	Stock	N.D.	N.D.	N.D.	N.D.	1.6	0.4	1.3	0.6	N.D.	N.D.	N.D.	N.D.

Sensitivities expressed in γ per milliliter.

C, complete inhibition; P, partial inhibition (few colonies or many tiny colonies).

—, Full growth in next dilution; N.D., not done.

*Reference strain, Streptococcus 98.

†Reference strain, *K. pneumoniae*, Type A, strain T.

‡Reference strain, *Esch. coli*, McLeod strain.

TABLE V. SENSITIVITY OF PATHOGENIC STRAINS OF PS. AERUGINOSA TO SEVEN ANTIBIOTICS

M.I.C. (γ/ML.)	PENICILLIN		STREPTOMYCIN		BACITRACIN		POLYMYXIN		AEROSPORIN		AUREOMYCIN		CHLOROMYCETIN	
	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL	NUMBER OF STRAINS	% OF TOTAL
2.5-3.1			1	0.5			1	4	18	72				
5-6.3	1	3	3	1.6			2	8	6	24	1	0.9		
10-12.5			10	5.4			12	48	1	4	2	1.7	1	4
25			29	15.7			9	36			2	1.7	0	
50			51	27.6			1	4			10	8.7	0	
100			50	27.0							23	20.0	0	
200			12	6.5							56	48.7	9	33
400-500			3	1.6							20	17.4	16	59
1,000											1	0.9	1	4
>1,000	32	97	26	14.1	25	100								
Total	33	100	185	100	25	100	25	100	25	100	115	100	27	100

M.I.C., minimum inhibiting concentration (complete).

DISCUSSION

Table VI is presented by way of summary and in order to bring out in a simple manner the comparisons of the activity of the different antibiotics against the organisms with which we are here concerned. This table shows the range of concentrations of each antibiotic that is required to inhibit the great majority of strains of proteus and pseudomonas. The values chosen for this purpose are those which completely inhibited at least two-thirds of the strains.

On the basis of all the data presented it is possible to arrange the seven antibiotics in the order of their effectiveness in vitro against these organisms. In the case of the strains of *B. proteus,* the most effective agents against the majority of the strains were chloromycetin and penicillin, but more than one-fourth of all the strains were resistant to the latter. Streptomycin and aureomycin ranked next, but rather high concentrations of these agents were necessary to produce inhibition and a significant proportion of the strains was highly resistant to streptomycin. Polymyxin, aerosporin, and bacitracin were essentially inactive.

TABLE VI. RANGE OF CONCENTRATION OF SEVEN ANTIBIOTICS REQUIRED TO INHIBIT MORE THAN TWO-THIRDS OF THE STRAINS OF B. PROTEUS AND PS. AERUGINOSA

	B. PROTEUS		PS. AERUGINOSA	
ANTIBIOTIC	M.I.C. (γ/ML.)	% OF STRAINS	M.I.C. (γ/ML.)	% OF STRAINS
Penicillin	12.5-50	68*	>1,000	97
Streptomycin	12.5-200	83†	12.5-200	82‡
Bacitracin	10,000+	100	>10,000	100
Polymyxin	>200	100	12.5-25	84
Aerosporin	>200	89	3.1-6.3	96
Aureomycin	100-400	92	50-400	95
Chloromycetin	12.5-50	79	200-400	92

M.I.C., minimum (complete) inhibiting concentration.
*28 per cent of the strains were resistant to > 500 γ per milliliter.
†11.4 per cent of the strains were resistant to > 500 γ per milliliter.
‡14 per cent of the strains were resistant to 1,000 γ per milliliter.

Against the strains of pseudomonas, aerosporin was by far the most effective with polymyxin ranking next. Streptomycin, aureomycin, and chloromycetin followed in that order with respect to their activity against the majority of the strains, but about 14 per cent of those tested with streptomycin were found to be highly resistant. Furthermore, the effective concentrations of aureomycin and chloromycetin as judged from these tests were rather high and for most of the strains were either close to or beyond the range of concentrations of these agents that are attainable even in the urine during systemic therapy.

For a discussion of a few of the many limitations of the type of in vitro data that have been presented here, the reader is referred to the previous paper of this series.[1] The comparisons with respect to the effectiveness of the different antibiotics against each of the organisms and with respect to each antibiotic against the different organisms are probably valid because of the uniform manner in which these tests were carried out. Within their limitations, however, these data should be of some practical value to those concerned with the choice and control of antibiotic therapy. In view of the varied metabolic activities of

different species of bacteria and the probable differences in the mechanisms by which the various antibiotics exert their antimicrobial action, it is hoped that data such as these may offer to the microbiologist and the biochemist useful hints as to the fundamental processes concerned in bacterial nutrition on the one hand and in antimicrobial action on the other.

SUMMARY AND CONCLUSIONS

The results of tests for in vitro sensitivity of a number of recently isolated pathogenic strains of *B. proteus* and *Ps. aeruginosa* to seven antibiotics have been presented.

The most effective agents against the majority of strains of *B. proteus* were chloromycetin, penicillin, streptomycin, and aureomycin, in that order. However, a significant proportion of the strains were highly resistant to penicillin and streptomycin, and rather high concentrations of aureomycin were required to inhibit most of the strains. Bacitracin was totally ineffective, and polymyxin and aerosporin were also essentially inactive. *B. proteus* was the only one among the common pathogenic gram-negative bacilli that were studied which was found to be resistant to polymyxin and aerosporin.

Against *Ps. aeruginosa*, aerosporin was the most effective and polymyxin ranked next. Streptomycin was active in moderately high concentrations against a majority of the strains, while a significant proportion were highly resistant to this agent. Aureomycin and chloromycetin were inhibitory in concentrations which, for most of the strains, are outside the range attainable clinically by systemic therapy. Penicillin and bacitracin were essentially without effect.

The authors are indebted to Marion E. Lamb and A. Kathleen Daly of the Bacteriological Laboratory of the Mallory Institute of Pathology for the isolation and identification of most of the strains, and to Janice M. Bryan for assistance in the isolation and identification of many strains and also in carrying out the sensitivity tests.

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THE TREATMENT OF PNEUMOCOCCIC PNEUMONIA WITH AUREOMYCIN

HARRY F. DOWLING, M.D., MARK H. LEPPER, M.D., HUGH H. HUSSEY, M.D., ESTON R. CALDWELL, JR., M.D., AND HAROLD W. SPIES, M.D.
WASHINGTON, D. C.

ALTHOUGH aureomycin* has been found to be effective in a wide variety of infections, the attention of most investigators has been directed toward those infections caused by viruses and rickettsiae. While it is true that another antibiotic, penicillin, is extremely effective in the treatment of pneumococcic infections, the relative value of aureomycin should be determined for several reasons. (a) There is a possibility that the low fatality rate obtained with penicillin therapy may not represent the lowest possible rate obtainable, and that better results may be obtained with aureomycin. (b) Aureomycin, if effective, can be used in patients who have developed hypersensitivity to penicillin. (c) Since the aureomycin can be detected constantly in the serum after it has been taken by mouth,[1] whereas this is not always true of penicillin administered orally, aureomycin may be preferable when oral medication is desired.

MATERIALS AND METHODS

We have determined the sensitivity of thirty-three strains of pneumococci obtained from patients with pneumonia and meningitis by inoculating tubes of tryptose-phosphate broth containing varying amounts of aureomycin, incubating for eighteen hours, and noting the first tube in which growth was not macroscopically evident. The sensitivity of these strains ranged from .007 to 0.312 μg of aureomycin per milliliter. Since serum concentrations of 1 to 4 μg of aureomycin per milliliter are obtained with the customary oral doses of aureomycin,[1] we treated 131 consecutive patients with typed pneumococcic pneumonia with aureomycin. During the time these patients were encountered, forty-three additional patients were treated who had typical pneumococcic pneumonia, as judged by the history, clinical course, physical and x-ray examinations, and leucocyte counts, although pneumococci were not obtained from the sputum or on blood culture. The results obtained in these 174 cases are the subject of the present paper.

After a specimen of sputum and of blood had been obtained for culture and typing, the patients were given either 250 mg. of aureomycin by mouth every three hours or 500 mg. at six-hour intervals until the temperature fell and remained within essentially normal limits for forty-eight to seventy-two hours. One patient had vomiting which was severe enough to prevent the absorption of sufficient aureomycin. She was given two intravenous doses of 500 mg. of aureomycin, but when vomiting persisted, a change was made to penicillin therapy.

The average dose of aureomycin administered per patient was 12 grams. In view of the fact that the patients treated earliest in the series were given aureomycin for a much longer time than the patients treated later, this average dose is much higher than we now consider necessary. At present, the average dose per patient is about 9 grams.

From the Health Department, District of Columbia, the George Washington and Georgetown Medical Divisions, Gallinger Municipal Hospital, and the Departments of Medicine, George Washington and Georgetown Universities.

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*Aureomycin was supplied by the Lederle Laboratories Division, American Cyanamid Company.

RESULTS

As shown in Table I, there were two deaths among the patients with typed pneumonia. One of these was a 58-year-old woman who died thirty minutes after a single dose of aureomycin. The other was an 85-year-old woman who had had hemiplegia for six months before developing pneumonia and was in an advanced stage of malnutrition on admission to the hospital. She died twenty hours after the first dose of aureomycin. Pneumococci were cultured from the blood of both patients.

TABLE I. RESULTS OF AUREOMYCIN TREATMENT OF PNEUMOCOCCIC PNEUMONIA ACCORDING TO AGE OF PATIENT

AGE (YR.)	PATIENTS FROM WHOSE SPUTUM AND/OR BLOOD PNEUMOCOCCI WERE RECOVERED		PATIENTS WITH TYPICAL PNEUMOCOCCIC PNEUMONIA FROM WHOM PNEUMOCOCCI WERE NOT RECOVERED	
	NUMBER	DIED	NUMBER	DIED
11-20	14	0	4	0
21-30	18	0	9	0
31-40	37	0	10	0
41-50	30	0	11	1
51-60	19	1	5	1
61-70	7	0	2	0
Over 70	6	1	2	0
Total	131	2	43	2

Among the patients from whom pneumococci could not be obtained, there were also two who died. Both were treated late in the disease. One was a 44-year-old Negro man whose treatment was started on the eighth day of disease and the other was a 55-year-old man who was given his initial dose of aureomycin on the ninth day of disease and who died after receiving only two doses of the drug.

From Table II it will be seen that the distribution of types was similar to that which is usually found in pneumococcic pneumonia. Bacteremia was detected in thirty-two (24 per cent) of the patients with typed pneumonia. Both of the deaths were in bacteremic patients. If the previously mentioned patient who was given penicillin when vomiting prevented the proper absorption of aureomycin is included with the patients who died as a treatment failure, there were three patients (2.3 per cent) who failed to improve with aureomycin, and all of them had bacteremia.

TABLE II. RESULTS OF TREATMENT OF PNEUMOCOCCIC PNEUMONIA ACCORDING TO TYPE OF PNEUMOCOCCUS

TYPE OF PNEUMOCOCCUS	ALL CASES		BACTEREMIC CASES	
	NUMBER OF PATIENTS	NUMBER DIED	NUMBER OF PATIENTS	NUMBER DIED
I	32	0	7	0
II	3	0	0	0
III	8	0	1	0
IV	6	0	0	0
VI	6	0	0	0
VII	16	0	7	0
VIII	4	0	1	0
Higher types	56	2	16	2
Total	131	2	32	2

Among 126 patients with typed pneumonia whose treatment was successful, the temperature in the case of 88 patients (or 70 per cent) had dropped below 101° in twenty-three hours or less from the time the first dose was given (Table III). Within forty-seven hours of the initial dose of aureomycin, the temperature of 116 (or 96 per cent) of the patients had fallen below 101° F. A similar rapid drop in temperature was observed in the patients from whom pneumococci were not obtained.

TABLE III. RAPIDITY OF CRISIS IN RECOVERED PATIENTS

TEMPERATURE OF 101° F. OR BELOW WITHIN:	TYPED CASES		NONTYPED TYPICAL PNEUMOCOCCIC PNEUMONIA CASES	
	NUMBER OF PATIENTS	PER CENT OF RECOVERED PATIENTS	NUMBER OF PATIENTS	PER CENT OF RECOVERED PATIENTS
0-23 hr.	88	69.8	24	61.5
24-47 hr.	33	26.2	9	23.1
48 hr. and over	5	3.9	6	15.3
Total	126	99.9	39	99.9

Complications (Table IV) were present in eight patients. Two had delayed resolution. One had a small pleural effusion. In one patient spread of the pneumonia was observed during treatment with aureomycin, but the pneumonia was eventually brought under control without increasing the dose of aureomycin. One patient, who had a relapse of the pneumonia as a result of vomiting of aureomycin and who improved after penicillin therapy was instituted, has already been mentioned. Another patient suffered a relapse nine days after aureomycin therapy had been discontinued. A second course of 250 mg. of aureomycin at three-hour intervals was successful in bringing about a prompt recovery. One patient was receiving Paritol* intramuscularly while his pneumonia was being treated with aureomycin. He developed, in the buttock

TABLE IV. COMPLICATIONS OF PNEUMOCOCCIC PNEUMONIA AND RESULTS OF TREATMENT WITH AUREOMYCIN

COMPLICATIONS	TYPED CASES	
	NUMBER OF PATIENTS	NUMBER DIED
Delayed resolution	2	0
Pleural effusion	1	0
Spread	1	0
Relapse	2	0
Pneumococcic abscess of buttock	1	0
Endocarditis	1	0
Total	8	0

at the site of one of these injections, an abscess from which pneumococci were cultured. This responded to incision and drainage and systemic treatment with aureomycin. Another patient experienced a drop in temperature under aureomycin treatment but later the fever returned, a tricuspid murmur appeared, and pneumococci were cultured from the blood. He was considered to have pneumococcic endocarditis and recovered after eight weeks of treatment with penicillin.

*Paritol, sulfated ester of dehydro-mannuronic acid.

DISCUSSION

Aureomycin was first used in pneumococcic pneumonia by Collins, Paine, and Finland,[2] who reported the successful treatment of two cases. Although the results obtained with penicillin in this disease have been better than those obtained with any therapeutic agent previously employed, nevertheless there is still an appreciable fatality rate in the upper age groups. We considered it worth while, therefore, to give aureomycin an extended clinical trial. The case fatality rate of 1.5 per cent which we obtained in 131 typed cases of pneumococcic pneumonia compares very favorably with a rate of 5.2 per cent among 686 patients treated in previous years in the same hospital with penicillin.[3] This is shown especially well in a comparison of patients, arranged according to age groups, treated by us with sulfonamides, penicillin, and aureomycin (Table V). The difference between the fatality rates in the penicillin-treated and the aureomycin-treated patients is not statistically significant, however, because of the small number of patients treated with aureomycin.

TABLE V. FATALITY RATES IN PATIENTS WITH PNEUMOCOCCIC PNEUMONIA (ARRANGED ACCORDING TO AGE OF PATIENTS) TREATED WITH SULFONAMIDES, PENICILLIN, AND AUREOMYCIN

AGE OF PATIENT (YR.)	SULFONAMIDES			PENICILLIN			AUREOMYCIN		
	NUMBER OF PATIENTS	DIED		NUMBER OF PATIENTS	DIED		NUMBER OF PATIENTS	DIED	
		NUMBER	PER CENT		NUMBER	PER CENT		NUMBER	PER CENT
12-20	147	4	2.7	52	1	1.0	14	0	0
21-30	311	10	3.2	143	1		18	0	
31-40	329	33	10.0	175	6	3.6	37	0	0
41-50	208	25	12.0	156	5		30	0	
51-60	136	35	25.7	64	8	15.1	19	1	6.3
61-70	82	24	29.3	52	9		7	0	
Over 70	37	15	40.5	36	6		6	1	
Age unknown	0	0		8	0		0	0	
All patients	1250	146	11.6	686	36	5.2	131	2	1.5

In the patients who recovered, the temperature dropped more rapidly than in our patients who received penicillin. Among the aureomycin-treated patients, 70 per cent had a temperature below 101° F. in less than twenty-four hours and 96 per cent within forty-eight hours; whereas among 262 patients treated with penicillin whose temperature drop was studied in this manner, the temperature fell below 101° F. within twenty-four hours in 52 per cent and within forty-eight hours in 77 per cent of cases.[4] When the patients treated with aureomycin are analyzed according to age, the presence of bacteremia, and the presence of associated diseases, they are found to be similar to those treated with penicillin. Accordingly, it appears that aureomycin is as effective as penicillin in the treatment of pneumococcic pneumonia, and there is a possibility that it may even be more effective. A larger number of patients must be treated before this can be determined with certainty.

SUMMARY

(1) Aureomycin has been used in the treatment of 174 patients with pneumonia. Among 131 patients with typed pneumococcic pneumonia there were

two deaths, and two deaths, also, among 43 patients with pneumonia which was presumably pneumococcic although no pneumococci were recovered.

(2) The fatality rate was lower and the temperature fell more rapidly in the patients treated with aureomycin than in 686 patients previously treated by us with penicillin.

(3) It is concluded that aureomycin is at least as effective as penicillin in pneumococcic pneumonia.

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STUDIES ON RHEUMATIC FEVER

Comparative Value of the Weltmann Serocoagulation Reaction and the Sedimentation Rate (Cutler) in Determining Activity of the Rheumatic Process

Harold G. Nelson, M.D.,* and Lieutenant Commander John R. Seal, Medical Corps, United States Navy†

ONE of the more difficult problems encountered in the clinical management of rheumatic fever is the determination of that point at which the rheumatic process can be judged to have become inactive. Frequently patients do not reach a clear-cut end point and continue to manifest one or more of the symptoms or findings which were present during the active stage for a prolonged period after all other evidences of activity have disappeared. The lack of an accurate objective yardstick by which to evaluate such patients and by which to determine the safety with which they may be allowed gradual resumption of their normal activities results in a considerable variation in the handling of these cases. Much depends on the facilities available as well as on the training, experience, and native caution of the physician concerned. The laborious, expensive, and time-consuming process of obtaining serial clinical, electrocardiographic, and laboratory observations has led to the search for reliable and easily performed laboratory tests which afford reasonably accurate evidence upon which a physician can base a decision and with which he can easily detect recurring rheumatic activity.

The sedimentation rate has come to be widely accepted as the single most reliable laboratory evidence of the presence or absence of rheumatic activity. Several observers, however, have sounded a note of caution in the placing of too much dependence on this test, pointing out the ease with which it is affected by many extraneous factors which are unrelated to the disease process. It has also been noted that in some patients the entire rheumatic cycle is attended by little or no increase in the sedimentation rate while in other patients the sedimentation rate has remained elevated for prolonged periods after all other evidences of rheumatic activity have disappeared and the course of the patient would indicate that true inactivity has been obtained. Our experience has paralleled that of others in this regard and the critical review of the records of many rheumatic fever patients at this hospital revealed that a too great dependence in the past on the sedimentation rate would appear to have condemned some of the patients to what probably were unwarranted periods of confinement to bed. The serum coagulation reaction, devised by Weltmann[1] in 1930, has been the subject of a number of reports[2] which have indicated that the reaction may have a considerable field of clinical usefulness in the differentiation between disease processes characterized by inflammation and exudation and those in which proliferation and fibrosis are predominant, although

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*Now at the A. B. Smith Clinic, Stillwater, Okla.

†Also participating in this study were the following personnel of Naval Medical Research Unit No. Four, formerly located at United States Naval Hospital, Dublin, Ga., where this study was conducted: Lt. Comdr. J. B. Black, M.C., U.S.N. (Chief of Medical Service), Lt. Comdr. R. L. Bailey, M.C., U.S.N., Lt.j.g. John S. Graettinger, M.C., U.S.N., Lt.j.g. Perry D. Cohn, M.C., U.S.N.R., and Lt.j.g. Lynn E. Anthony, M.C., U.S.N.

the sedimentation rate may be increased in either. These reports have also indicated that the Weltmann reaction is little influenced by allergic and metabolic disorders, anemia, salicylates,[3] or other factors which are known to sometimes alter the sedimentation rate. Dees,[4] in 1940, summarized the literature on the reported results of the Weltmann reaction and reported her findings in 246 patients with a variety of disorders, the majority of whom were children with rheumatic fever or acute appendicitis. Her results were in agreement with those of previous workers, namely: in diseases characterized by inflammation and exudation there was a "shift to the left" of the reaction (coagulation of the serum occurring only with higher concentrations of electrolyte); in those characterized by fibrosis or proliferation there was a "shift to the right"; and in normal individuals, those with allergic and metabolic disorders, blood dyscrasies, and chronic arthritis there was a constant reaction in the midzone ("normal").

Scherlis and Levy,[5] investigating the relationship of the Weltmann reaction and the erythrocyte sedimentation rate (Wintrobe) in patients with active rheumatic fever and rheumatic heart disease, concluded that in a single determination a normal coagulation band and a normal sedimentation rate were equally accurate (93.9 and 91.1 per cent respectively) in indicating the absence of active disease, but that an abnormal Weltmann reaction, especially a shortened coagulation band ("shift to the left"), was more frequently associated with disease activity than was an elevated sedimentation rate. They were unable to attach any prognostic significance to a persistent "shift to the right" of the Weltmann reaction in the development of rheumatic heart disease within the interval their patients remained under observation. These results were in agreement with an earlier study by Klein, Levinson, and Rosenblum[6] of children with rheumatic fever. These authors found that the Weltmann reaction reflected the various stages of evolution of the pathologic changes of rheumatic fever, that it tended to return to normal prior to the sedimentation rate when the process became inactive, and that it was of considerable complementary value to the sedimentation rate. Furthermore, they noted the value of the Weltmann test in the differentiation of subacute bacterial endocarditis from active rheumatic carditis.

The relatively large number of rheumatic fever patients available at the United States Naval Hospital, Dublin, Ga., presented us with an opportunity to conduct an extensive study of the Weltmann reaction and to compare its value with that of the erythrocyte sedimentation rate. By comparing the clinical status of the individual patients with the results of the sedimentation rate and the Weltmann reaction at specified intervals over a relatively long period of time, it was sought to determine which test was of more value as a criterion of rheumatic activity and the complementary value of routinely performing both tests on patients with rheumatic fever.

MATERIAL AND METHODS

Between November, 1946, and January, 1948, 400 patients with a diagnosis of rheumatic fever had a sufficient number of serial Weltmann reactions performed to warrant their inclusion in this study. Patients who were in the hospital at the inception of this study and who were expected to require several months of further hospitalization were included regardless of the stage of their disease, and others, transferred to this hospital during the study period, were added when the workload permitted. Since patients with rheumatic fever were usually retained at the hospital to which they were initially admitted until it was considered safe for them to travel, the majority had attained an inactive phase of the disease prior to transfer and had no recurrences while under our observation.

These patients were all white men between 18 and 25 years of age transferred to this hospital for convalescence. The selection of patients for inclusion in the study of the case of those patients present in the hospital at its inception was predicated only upon their probable availability over a considerable period of time. Selection of those patients transferred

to the hospital after the beginning of the study was automatic, all being nominated for inclusion, and the laboratory continued observations upon as many as the workload would allow in the order of their nomination. All patients who had less than four observations remaining after all deletions were dropped from the study and no results from such patients appear in the totals.

Fifty of the 400 patients were classified as showing clinical evidences of activity according to the criteria defined below, usually only during the initial period of observation in those admitted during the course of the study. A few patients had recurrent polycyclic activity throughout. The remaining 350 patients presented no evidences of clinical activity of the rheumatic process at any time during their period of observation.

Criteria for Determining Clinical Activity of Rheumatic Fever.—Herein lay the most difficult problem presented by the study. The extreme variability of symptoms and findings which can be accepted as indicating that rheumatic activity of some degree continues is such that the adoption of a single criterion is impossible. It is agreed that following an attack of rheumatic fever, continued low-grade fever, tachycardia, weight loss or failure to gain on an adequate diet, anorexia, pallor, skin nodules, recurrent erythema marginatum, persistent or intermittent arthralgia, changing cardiac findings, and instability of the electrocardiogram are among the more common evidences that the rheumatic process has not yet reached an inactive phase (provided no other cause can be found for the persistence of one or more of these findings). Less tangible but not to be ignored in a military hospital if one wishes to obtain the maximum information concerning a patient's clinical course is the opinion of an experienced observer who has had the opportunity to watch the patient over a considerable period of time and is familiar with his behavior on the ward when he is not aware of being directly under scrutiny.

Fortunately, prior to the inception of this study, an attempt had been made by the medical service to provide a systematic method of weekly evaluation of rheumatic fever patients for evidences of activity in order to allow the safe assignment of all patients to rehabilitation activities according to their degree of convalescence. A standardized plan, known locally as the Dublin Plan, had been adopted early in 1946 and published in chart form on all rheumatic wards. All medical officers engaged in the care and treatment of rheumatic patients were required to familiarize themselves with the classification of patients set forth by this plan, and detailed instructions were provided therein for the clinical and laboratory criteria to be used in classification as well as the activities allowed and the treatment program to be followed. Strict bed rest was enforced and oral salicylate therapy (125 grains per 24 hours) was routinely given during the early phase of the disease. Abatement of the clinical phenomena and trends of the weekly electrocardiograms, leucocyte counts, and sedimentation rates toward normal allowed an increase in the recreational activities in bed, but a patient was not allowed to become ambulatory until all evidences of activity had disappeared and had remained absent for at least one week after the cessation of salicylate therapy. Ambulation followed a carefully regulated program of gradually increasing activity until full physical activity had been resumed prior to the patient's release from the hopsital. The plan required continuation of careful clinical and laboratory observations throughout this entire period for early detection of recurrence of rheumatic activity, should this occur.

All medical officers were required to submit a list of their patients by classification to the Chief of Medicine once weekly. Each medical officer was thus charged with the responsibility of weighing all of the evidence of rheumatic activity in each of his patients at no more than weekly intervals. Discrepancies or unusual trends in the patient's course were normally cause for consultation between the Chief of Medicine and the patient's ward medical officer.

Experience gained through the operation of this program had qualified the majority of the ward medical officers in the accurate interpretation of evidences of rheumatic activity and most had learned to disregard a persistent elevation of the sedimentation rate in making their evaluations. For this reason it was determined that the most reliable evidence which could be used in evaluating the Weltmann coagulation reaction and the sedimentation rate would be the ward medical officer's opinion as to the status of his patient at the time the blood was drawn for performance of these tests. It was recognized that his opinion might

be influenced by the results of the tests done the previous week which could not be withheld for obvious reasons, and in order to minimize bias from this source each patient's chart was later reviewed with the cognizant medical officer and wherever the chart did not contain supportive entries to substantiate his opinion that the patient had been "active," the results were deleted for the week in question.

Technique of the Weltmann Reaction.—Stock solutions were prepared by the addition of the following quantities of a 10 per cent solution of calcium chloride hexahydrate* to a series of ten 500 c.c. glass-stoppered volumetric flasks: 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, and 0.5 c.c. Each flask was then numbered in order from one to ten, filled to the 500 c.c. mark with distilled water, and thoroughly mixed. Each stock solution thus contained the final concentration of calcium chloride indicated below:

Flask	1	2	3	4	5	6	7	8	9	10
Per cent Conc. $CaCl_2$	0.10	0.09	0.08	0.07	0.06	0.05	0.04	0.03	0.02	0.01

To perform the test, 0.1 c.c. of nonhemolyzed patients serum was placed in each of ten 13 by 100 mm. test tubes which were serially numbered and placed in a rack in order from one to ten. To each test tube were added 5 c.c. of the correspondingly numbered stock solution; the individual tubes were vigorously shaken by hand, returned to the rack, and the rack was immersed in a boiling water bath to a depth equal to two-thirds of the height of the tubes. After fifteen minutes the tubes were removed from the boiling water and read immediately. A control serum with a normal value was routinely run on each day the Weltmann test was performed.

Reading the Test: The last tube showing definite coagulation was read as the coagulation band. If there was a definite coagulum (flocculation) in the next tube but the menstruum was not clear, the reading of one-half was used; thus if the first six tubes showed complete coagulation and the seventh tube showed partial coagulation, the coagulation band was read as 6.5. Weltmann reported that normal serum gives a coagulation band of 6. If the coagulation occurred in Tubes 7, 8, 9, and 10 (band is greater than 6), there is a "shift to the right"; conversely a coagulation of less than 6 represents a "shift to the left."

In our routine work we eventually utilized what we have termed "the preliminary Weltmann" which consisted of using only Tubes 4 to 8 inclusive. If coagulation failed to occur in Tube 4, Tubes 1, 2, and 3 were then run. If coagulation occurred in all tubes including Tube 8, then Tubes 9 and 10 were run.

Technique of the Erythrocyte Sedimentation Rate.—The Cutler method, being the most commonly used method in Naval hospitals, was continued routinely throughout the period of this study. Five-tenths cubic centimeter of 3.8 per cent sodium citrate was placed in a 5 c.c. Cutler sedimentation tube and freshly drawn blood was added to bring the column to the zero mark. The tube was inverted six times, placed in a vertical position in a metal rack, and read at the end of one hour. Any reading above 10 mm. per hour was considered to be an abnormal or increased sedimentation rate. In the event that a patient had an anemia or whenever there was continued disagreement between the patient's course and the Cutler results, the method of Wintrobe and Landsberg[7] was employed concomitantly with the Cutler method.

RESULTS

Statistical Analysis of Derived Data.—The data from each patient was entered weekly onto a large chart. The presence or absence of clinical activity was indicated simply by the insertion of a plus or zero sign and the actual results of the sedimentation rate and Weltmann reaction were entered opposite this under the appropriate week of illness. As the object of the study was to compare the two tests with the state of activity of the rheumatic process ad-

*$CaCl_2.6H_2O$ Merck. Use of the hexahydrate is essential to the correct performance of this test.

judged clinically, either or both of the tests were given credit for being in error when the results were in disagreement with the entry under clinical activity. Thus if the entry under clinical activity was a plus sign, the Weltmann reaction had been read as showing coagulation to be complete in the sixth tube (normal), and the sedimentation rate was 23 mm. per hour for a given week, the patient was credited with a Weltmann error for that week. In those patients who exhibited no evidences of activity throughout their course and on whom observations had been begun immediately upon admission or continued until disposition, the first or last three weeks' observations were deleted from the results prior to statistical analysis in order to eliminate errors which might be introduced by subsiding activity or preceding recurrent activity about which we had inadequate information. After all deletions the 400 men had a total of 2,552 observations remaining for an average of 6.4 observations per man. As might be expected those who exhibited activity while under observation had more observations made because of their longer retention in the hospital and the 50 men in this category had a total of 695 observations remaining for an average of 13.9 observations per man. The 350 men who exhibited no activity averaged 5.3 observations per man.

The results obtained on the entire series of 400 patients are shown in Table I.

TABLE I. DISCREPANCIES BETWEEN WELTMANN REACTION, SEDIMENTATION RATE, AND THE CLINICAL STAGE OF ACTIVITY IN 400 RHEUMATIC FEVER PATIENTS

	TOTAL		ERRORS			
			NUMBER		PER CENT	
TEST	MEN	OBSERVATIONS	MEN	OBSERVATIONS	MEN	OBSERVATIONS
Weltmann	400	2,552	47	71	11.8 ± 1.61	2.8 ± 0.33
Cutler	400	2,552	136	343	34.0 ± 2.37	13.4 ± 0.21

It is seen from Table I that the Weltmann test failed to correlate with the clinical status 71 times, whereas the sedimentation rate yielded 343 discrepancies. We thought it of interest to analyze the results in detail according to whether or not the patient had rheumatic activity. Table II shows the results obtained in the 50 patients who showed clinical activity while they were under observation.

TABLE II. DISCREPANCIES BETWEEN WELTMANN REACTION, SEDIMENTATION RATE, AND THE CLINICAL STAGE OF ACTIVITY IN THE 50 PATIENTS EXHIBITING ACTIVE RHEUMATIC FEVER DURING STUDY

	TOTAL		ERRORS			
			NUMBER		PER CENT	
TEST	MEN	OBSERVATIONS	MEN	OBSERVATIONS	MEN	OBSERVATIONS
Weltmann	50	695	20	35	40 ± 6.9	5 ± 0.8
Cutler	50	695	32	82	64 ± 6.8	12 ± 1.2

One of the first things that should be pointed out is the difference in the number of men incurring errors as designated by the results of the two tests. When the observations are considered and using patients as a unit, only 20, or 40 per cent of the men contributed discrepancies from the Weltmann, whereas 32, or 64 per cent, were considered as having errors from the Cutler. This means that the ward medical officer could, from this particular sample of patients, have accepted the Weltmann test results alone and been correct for three-fifths of the men. The Cutler would have given consistent results for approximately two-fifths of the men.*

Of the 20 men having incorrect Weltmann results, 16 also showed up as being recorded with Cutler errors. This would leave 4 men having Weltmann errors only. Of the 32 men for whom aberrant Cutler results were recorded, 16 did not show Weltmann errors. The former group (4 men) contributed five errors (three of these errors occurred or were recorded for the last week of activity) and the latter group (16 men) contributed twenty-eight errors.

There were 695 observations made on the 50 men included in the study or an average of about 14 observations per man. There was a minimum of one week between recorded observations, although this interval was two or three weeks in some cases. The maximum number of observations was 35, with 8 being the smallest number for any individual.

Of the 695 observations, 35, or 5 per cent, of the Weltmann results were shown as not coinciding with the clinical status and 82, or 12 per cent, of the sedimentation rates disagreed with the ward medical officers' decisions as to the clinical status (this difference could be attributed to chance less than 1 time in 100).

Table III shows the relationship of the Weltmann and Cutler results when limited to those weeks when the patient was judged to be clinically active.

TABLE III. DISCREPANCIES BETWEEN WELTMANN REACTION, SEDIMENTATION RATE, AND CLINICAL STATUS IN 50 RHEUMATIC FEVER PATIENTS WHEN OBSERVATIONS ARE RESTRICTED TO ACTIVE STAGE ALONE

TEST	NUMBER OF OBSERVATIONS	ERRORS			
		NUMBER		PER CENT	
		MEN	OBSERVATIONS	MEN	OBSERVATIONS
Weltmann	281	17	29	34 ± 5.3	10 ± 1.8
Cutler	281	15	54	30 ± 5.1	10 ± 2.4

There were 17 men (34 per cent) who produced Weltmann errors and 15 men (30 per cent) representing Cutler disagreements. This small difference would be attributed to sampling errors. Twelve of the 17 men producing Weltmann errors did not show Cutler errors and 10 of the 15 patients for whom Cutler errors were recorded produced no Weltmann errors. There were 26 different individuals that produced either Weltmann or Cutler errors or both (when the observations are considered, twice as many errors were recorded for the Cutler as for the Weltmann). This is a significant difference;

*The indications are, on repeated samples, that the Weltmann would vary between 81 and 41 per cent correct while the Cutler would fluctuate between 57 and 15 per cent.

however, there are apparently some individual patient differences that influence the results. In the first place it may be seen in Table III that 17 men contributed 29 Weltmann errors while at the same time only 15 men produced 54 Cutler errors. This is an average of 1.7 Weltmann errors per individual, while the Cutler average is 3.6 per individual or over twice as many. Upon re-examining the original data, it was found that 4 individuals had mutual errors, i.e., Weltmann and Cutler results both aberrant. Upon closer inspection it looked as if 4 individuals (Patients 23, 24, 40, and 48) might have been influenced by other factors and there is still some doubt whether they belonged in the same population as the other 46 patients.

In Table IV is a summary of the test results in these patients, one of whom died of active rheumatice fever; the other three were invalided from service with valvular heart disease.

TABLE IV. UNUSUALLY HIGH INCIDENCE OF DISCREPANCIES BETWEEN LABORATORY RESULTS AND CLINICAL STATUS IN 4 PATIENTS WITH ACTIVE RHEUMATIC FEVER*

NUMBER OF OBSERVATIONS		CUTLER ERRORS		WELTMANN ERRORS		MUTUAL ERRORS	
TOTAL	ACTIVE	NUMBER	PER CENT	NUMBER	PER CENT	NUMBER	PER CENT
85	67	32	42	14	18	12	16

*One died of active rheumatic fever; 3 were discharged with valvular heart disease.

These four patients had a total of 85 observations or an average of 21.2 as compared with the over-all average of 13.9. Furthermore, 67, or 79 per cent, of all the observations were made while the patients were in a clinically active stage. It may be noted that 42 per cent of all the Cutler results were divergent, as opposed to a discrepancy of 18 per cent in the Weltmann results. These may be compared with all patients included in Table III where only 10 per cent Weltmann and 19 per cent Cutler errors were reported.

In view of the foregoing comments it seemed appropriate to present in Table V an abridged version of Table III with all data relating to Patients 24, 34, 40, and 48 deleted.

TABLE V. DISCREPANCIES BETWEEN WELTMANN REACTION, SEDIMENTATION RATE, AND CLINICAL STATUS IN REMAINING 46 PATIENTS WITH ACTIVE RHEUMATIC FEVER AFTER DELETION OF THE 4 PATIENTS IN TABLE IV

TEST	NUMBER OF ERRORS	PERCENTAGE OF ERRORS
Weltmann	15	7 ± 1.7
Cutler	22	10 ± 2.3

As may be noted from Table V, the per cents of error of the Weltmann and Cutler tests approach each other when the data contributed by the four patients mentioned are not included. The number of men involved would be 13 for the Weltmann and 11 for Cutler (four less for each test than shown in Table III) and this would mean about one Weltmann error and two Cutler errors for each individual for whom errors were reported.

While the discussion is still limited to the active periods, it might be well to introduce the other important variable—time. Table VI provides information concerning the terminus of the active cycle.

TABLE VI. DISCREPANCIES BETWEEN WELTMANN REACTION, SEDIMENTATION RATE, AND CLINICAL STATUS IN OBSERVATIONS MADE DURING LAST 4 WEEKS OF ACTIVITY ON 47 RHEUMATIC FEVER PATIENTS*

WEEK OF ACTIVITY	NUMBER OF OBSERVATIONS	ERRORS			
		NUMBER		PER CENT	
		WELTMANN	CUTLER	WELTMANN	CUTLER
	Total 171	16	19	9	11
Last week	63	12	12	19	19
Next to last week	44	3	3	7	7
2nd to last week	32	0	1	0	3
3rd to last week	22	1	3	5	14

*Data from 3 patients omitted because of their transfer elsewhere while still active.

Each test had twelve errors recorded in the last week of the active cycle. This was 19 per cent of the observations for the week. The 19 per cent error was the same as shown in Table III for the Cutler and about twice the total active error rate for the Weltmann.

There was a total of twenty-six active stage Weltmann errors and forty Cutler errors for the 47 patients included in Table V. In other words, 46 per cent of the Weltmann and 30 per cent of the Cutler discrepancies were recorded in the final week of the clinically active stage. It is obvious that there is a decided grouping of the errors just prior to the time the patient's clinical activity disappears.

Practically all of the comments made previously have been limited to observations made while the patients were considered to be clinically active. Since the greater part of the total observations was made during the inactive stages, Table VIII is presented to give information regarding the results during this phase.

TABLE VII. DISCREPANCIES BETWEEN WELTMANN REACTION, SEDIMENTATION RATE, AND CLINICAL STATUS IN 50 PATIENTS AFTER TERMINATION OF ACTIVE RHEUMATIC FEVER

TEST	TOTAL		ERRORS			
			NUMBER		PER CENT	
	MEN	OBSERVATIONS	MEN	OBSERVATIONS	MEN	OBSERVATIONS
Weltmann	50	414	6	6	12 ± 4.6	1 ± 0.5
Cutler	50	414	19	28	38 ± 6.8	7 ± 1.3

By referring to Table II it may be seen that there were 695 total observations made on the 50 patients. Table VII reveals that 414 (60 per cent) of the total observations were made while the patients were judged by the ward medical officers to be clinically inactive. From Table III a direct comparison may be made on the test results for the active and inactive stages. The Weltmann errors involved 34 per cent of the men in the active period against 12 per cent (Table VII) during the inactive period (the probability of this happening by chance is less than 1 in 100). There was also a significantly smaller percentage of observation errors for this test in the inactive period than in the clinically active stage—1 per cent as compared to 10 per cent. There was

also a significantly smaller percentage difference in the Cutler observation errors. During the active interval 19 per cent of the Cutler observations were aberrant as compared with only 7 per cent of the inactive results.* However, instead of the smaller number of men, there were actually more (10 to 15) related to Cutler errors in the inactive period than in the clinically active intervals. It becomes obvious that the chances of an observation being in error are much greater while the patients are clinically active. When the comparison of the test is restricted to only the inactive period, the Weltmann errors involve only 12 per cent of the men as contrasted to 38 per cent for the Cutler test. When observations only are considered, only 1 per cent of the Weltmann test results were recorded as incorrect while 7 per cent of the sedimentation rates were abnormal. It would appear that the Weltmann is significantly more reliable (correlates with the clinical status) than the Cutler when rheumatic fever patients are clinically inactive.

There is perhaps one additional question that should be answered—When did the observation errors occur, especially the Cutler errors? It has been mentioned previously that many of them happened in the first normal week. Table VIII provides a breakdown of the observations for both the Weltmann and Cutler tests during the first four weeks of the inactive periods.

TABLE VIII. DISCREPANCIES BETWEEN WELTMANN REACTION, SEDIMENTATION RATE, AND CLINICAL STATUS DURING FIRST 4 WEEKS OF INACTIVITY FOLLOWING ACTIVE RHEUMATIC FEVER

WEEK OF INACTIVITY	NUMBER OF OBSERVATIONS	ERRORS			
		NUMBER		PER CENT	
		WELTMANN	CUTLER	WELTMANN	CUTLER
First week	63	0	12	0	19
Second week	41	0	4	0	10
Third week	35	1	2	3	6
Fourth week	27	0	0	0	0
Total	166	1	18	1	11

Eighteen Cutler observation errors occurred during the first four weeks of the clinically inactive period. Of these, twelve, or 43 per cent of the twenty-eight total inactivity discrepancies, occurred during the first week following the active periods. As has been mentioned, there were also twelve observation (Table VI) errors in the last week of the active periods. However, upon checking, it was found that the same individuals were not involved.

The results obtained in the 350 patients who did not exhibit clinically active rheumatic fever while under observation are found in Table IX.

In going over the patients' records it was found that there were 22 patients who had four or more abnormal sedimentation rates during the course of our observation. Out of a total of 254 observations on these patients, there were 136 observations in which the sedimentation rate was considered abnormal, i.e., above 10 mm. per hour. These patients did not have an anemia

*If the comparison were made with only the 46 men included in Table V, the observed percentage difference in Cutler errors could easily have resulted by chance.

TABLE IX. DISCREPANCIES BETWEEN WELTMANN REACTION, SEDIMENTATION RATE, AND CLINICAL STATUS IN 350 PATIENTS WHO DID NOT EXHIBIT ACTIVE RHEUMATIC FEVER DURING STUDY

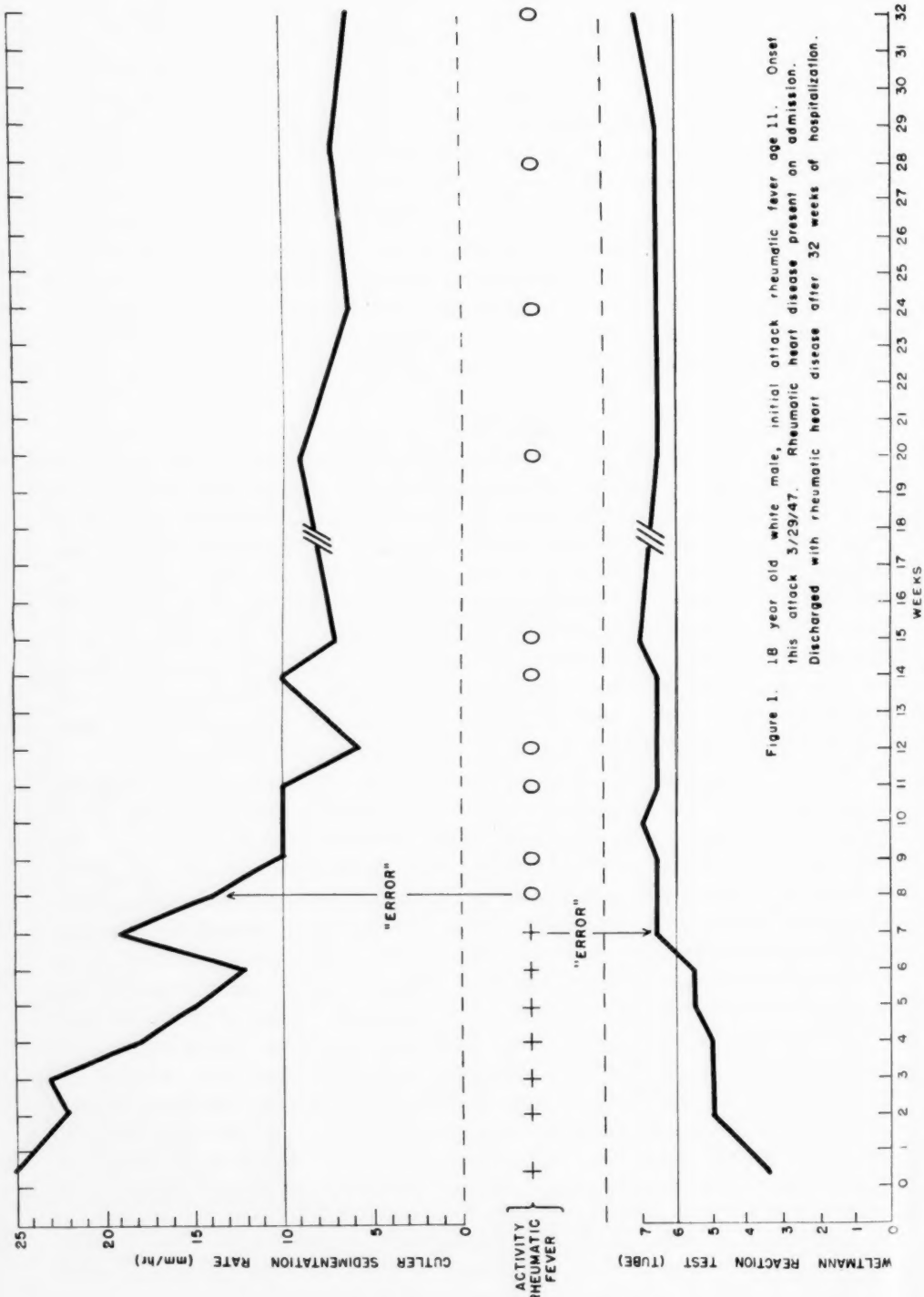
TEST	TOTAL		ERRORS			
			NUMBER		PER CENT	
	MEN	OBSERVATIONS	MEN	OBSERVATIONS	MEN	OBSERVATIONS
Weltmann	350	1,857	27	36	7.7 ± 1.42	1.9 ± .32
Cutler	350	1,857	104	261	29.7 ± 2.46	14.1 ± .81

and there was no evidence of clinical activity. If these 22 patients are excluded from the 350, the percentage of Cutler values which does not correlate with the clinical findings is 7.8. This figure is similar to the 7 per cent disagreement found during the inactive period of the 50 patients with rheumatic fever who had clinical activity during this study (Table VII).

DISCUSSION

The foregoing statistical analysis of the data derived in this study has indicated that both tests correlated to a high degree with the selected criterion for clinical activity of the rheumatic fever, the Weltmann reaction being more often in agreement than the sedimentation rate. Furthermore, the analysis indicated that at certain periods in the activity cycle of the rheumatic process there was a tendency for greater numbers of discrepancies to occur (Tables VI and VIII) and that certain patients contributed more than their share of discrepancies in the correlation between these tests and the state of clinical activity (Tables IV and IX). Recognition of these two sources of error allows one to place even a higher degree of faith in the results of the two tests in most patients, particularly when they were used in conjunction.

It was expected that a greater percentage of errors or discrepancies would be seen in the transition zone between activity and quiescence of the rheumatic process because of the inherent difficulty in placing an exact end point within a narrowly defined time interval on the interpretation of clinical activity. If errors were introduced by faulty clinical appraisal alone during this period, it might be expected that they would occur equally in both tests and this appears to occur in the analysis of the errors occurring during the last two weeks of activity (Table VI). However, the results for the first two weeks of inactivity (Table VIII) are quite different and involved only errors in the sedimentation rate. At first glance this might be interpreted as indicating that the Weltmann reaction results influenced the medical officers opinion and introduced a large factor of bias, but the results presented in Table VII are against any such interpretation and in our experience the errors are largely due to the earlier return of the Weltmann reaction to normal values, often occurring prior to the disappearance of clinical evidences of activity and being of some prognostic value, whereas in a number of patients the sedimentation rate declined to normal values one or two weeks following the disappearance of clinical activity. Fig. 1 illustrates this phenomenon and, as can be seen, this one patient introduced two errors in the Weltmann


CUTLER SEDIMENTATION RATE (mm/hr)
25
20
15
10
5
0
"ERROR"
ACTIVITY RHEUMATIC FEVER
+ + + +

reaction results during the final two weeks of activity and one error in the sedimentation rate during the initial week of inactivity. Fig. 2, depicting a somewhat more complicated course in an indivdual with pericarditis and rheumatic pneumonitis who had a recurrent pericarditis following tonsillectomy, illustrates not only this source of error but also presents in linear form the behavior of the Weltmann reaction during some of the more exudative manifestations of the rheumatic process.

With a few exceptions (Table IV) the errors introduced by the occurrence of multiple discordant observations in the same individual involved mainly the sedimentation rate and fell into two general patterns. Certain patients failed to exhibit increases in the sedimentation rate at any time while under observation although adjudged "active" by their medical officer on the basis of electrocardiographic instability, clinical carditis, or other evidence. Such a patient is illustrated in Fig. 3 and this one patient introduced thirteen errors in the sedimentation rate and seven errors in the Weltmann reaction during a 16-week period. One might question the validity of the clinical interpretation in such a patient, but the persistence of an unstable and prolonged P-R interval coupled with changing cardiac findings throughout the period indicated by plus marks and later stability of these findings cannot be appraised otherwise.

The second type of patient encountered who introduced multiple errors was somewhat more frequent than the type illustrated in Fig. 3 and consisted of those individuals who exhibited a persistent elevation of the sedimentation rate after all other evidences of activity had disappeared. Fig. 4 illustrates such a patient. Eight of ten sedimentation rates (plus two Wintrobe corrected sedimentation rates) performed over a 24-week period were in error as compared with no errors in the Weltmann reaction. Patients of this type contributed a large share of the errors in Table IX, as is noted in the comments concerning that table. Patients exhibiting persistently abnormal Weltman reactions when the clinical appraisal was "inactive" and the sedimentation rate was normal were not encountered, and in our experience one of the more outstanding values of the Weltmann lay in its assistance in the evaluation of such cases with persistently elevated sedimentation rates.

Over and above the value of the Weltmann reaction indicated in the statistical tabulation of its accuracy in rheumatic fever is its ability to provide information of importance in differential diagnosis. The following case report illustrates one such usage.

A. S. E., an 18-year-old white man, had a past history of rheumatic fever at the age of 11. He was transferred to this hospital twenty weeks after the onset of carditis and pericarditis following a respiratory infection in October, 1946. Upon admission he was found to be weak, undernourished, anemic, and mildly dyspneic. The patient had low-grade fever and tachycardia, subcutaneous nodules over the elbows, and an enlarged heart with the murmurs of aortic and mitral insufficiency. Upon a regime of bed rest, salicylates, and restricted salt intake he improved somewhat with an increase in the vital capacity from the admission level of 1,500 c.c. to 2,300 c.c. by the thirty-first week. He was still considered to be "active" and the diagnosis of subacute bacterial endocarditis continued to be strongly

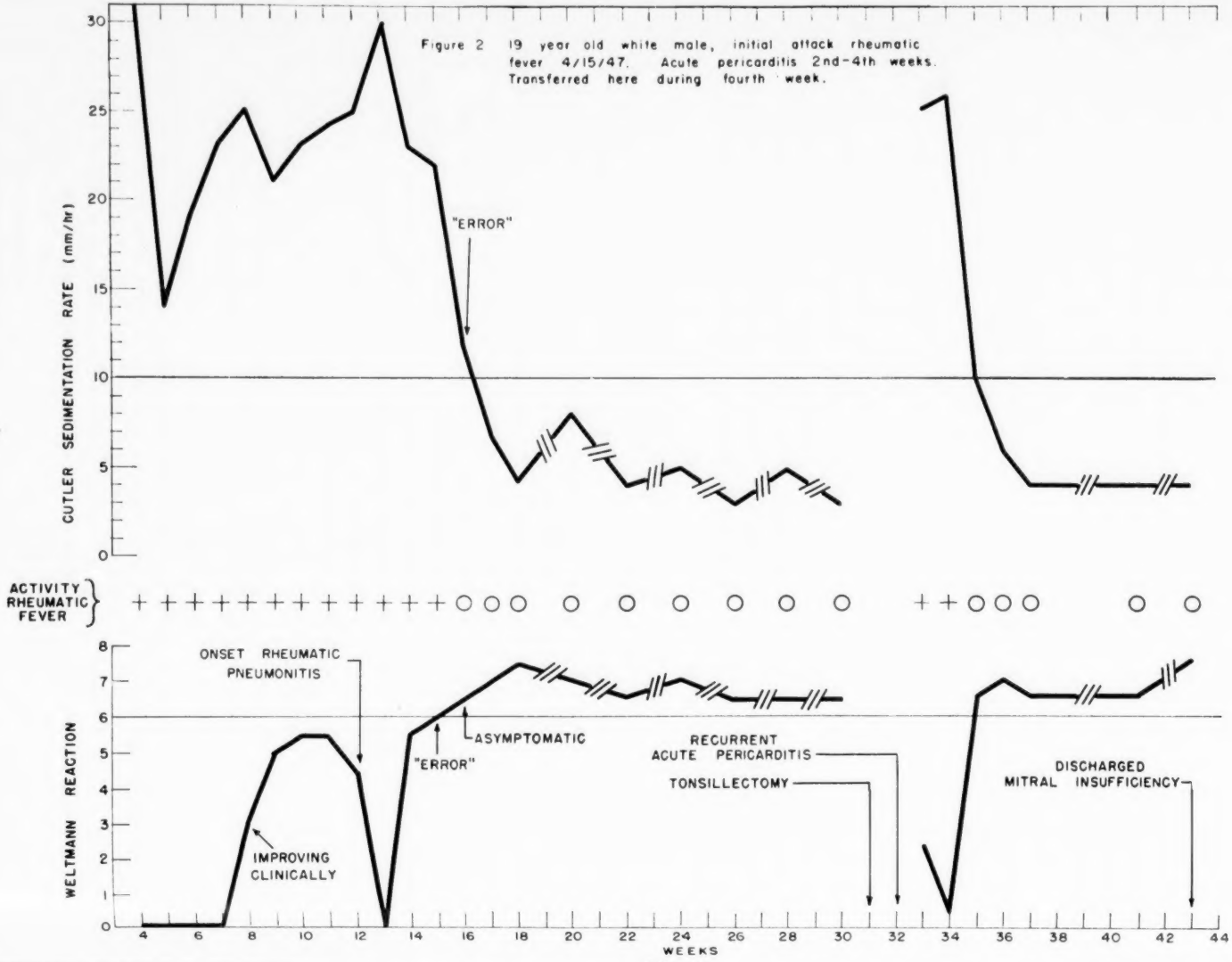


Figure 2 19 year old white male, initial attack rheumatic fever 4/15/47. Acute pericarditis 2nd-4th weeks. Transferred here during fourth week.
CUTLER SEDIMENTATION RATE (mm/hr)
"ERROR"
ACTIVITY RHEUMATIC FEVER
WELTMANN REACTION
ONSET RHEUMATIC PNEUMONITIS
IMPROVING CLINICALLY
"ERROR"
ASYMPTOMATIC
RECURRENT ACUTE PERICARDITIS
TONSILLECTOMY
DISCHARGED MITRAL INSUFFICIENCY
WEEKS

Fig. 2.

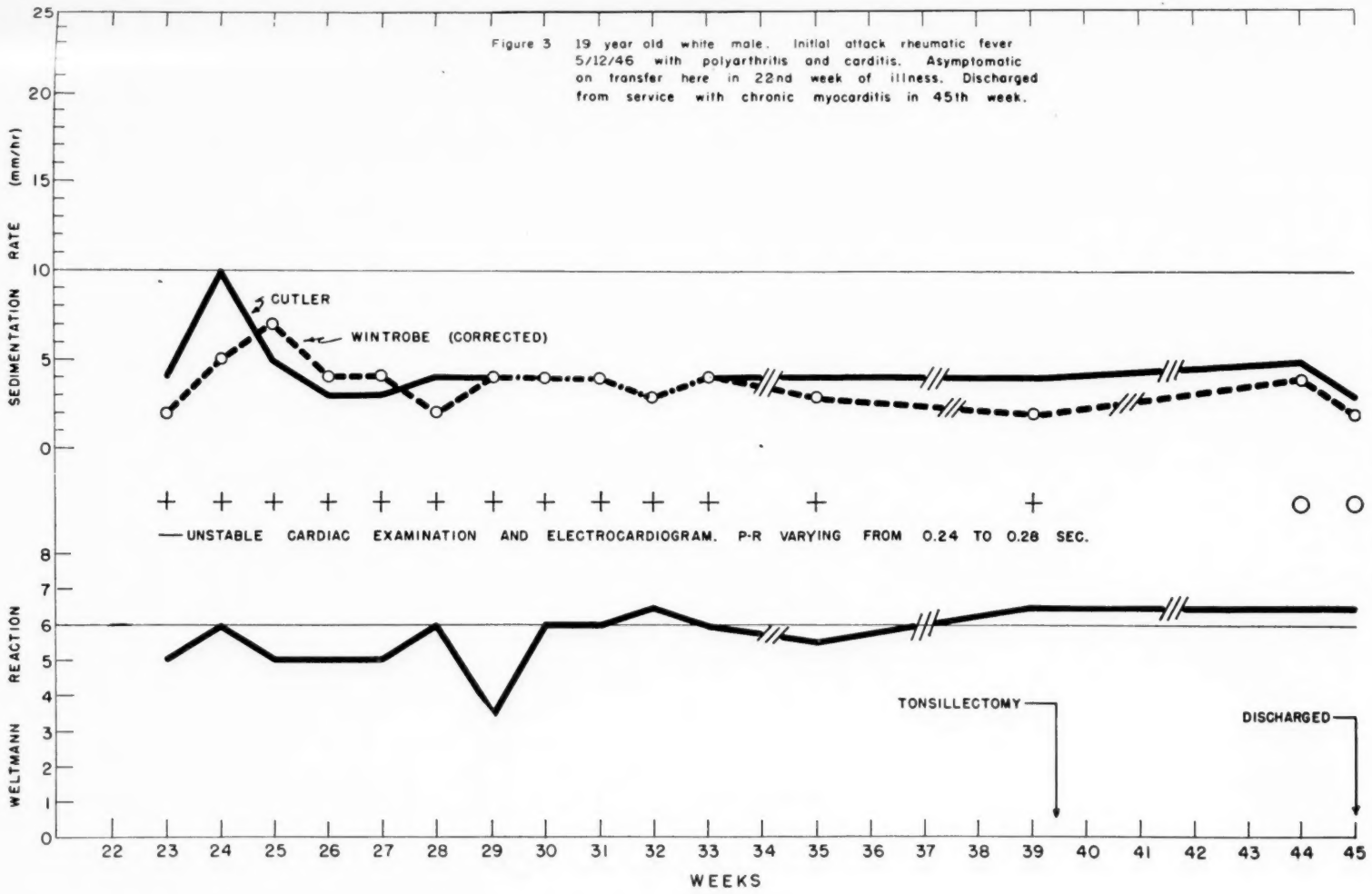


Figure 3 19 year old white male. Initial attack rheumatic fever 5/12/46 with polyarthritis and carditis. Asymptomatic on transfer here in 22nd week of illness. Discharged from service with chronic myocarditis in 45th week.
SEDIMENTATION RATE (mm/hr)
CUTLER
WINTROBE (CORRECTED)
UNSTABLE CARDIAC EXAMINATION AND ELECTROCARDIOGRAM. P-R VARYING FROM 0.24 TO 0.28 SEC.
WELTMANN REACTION
TONSILLECTOMY
DISCHARGED
WEEKS

Fig. 3.

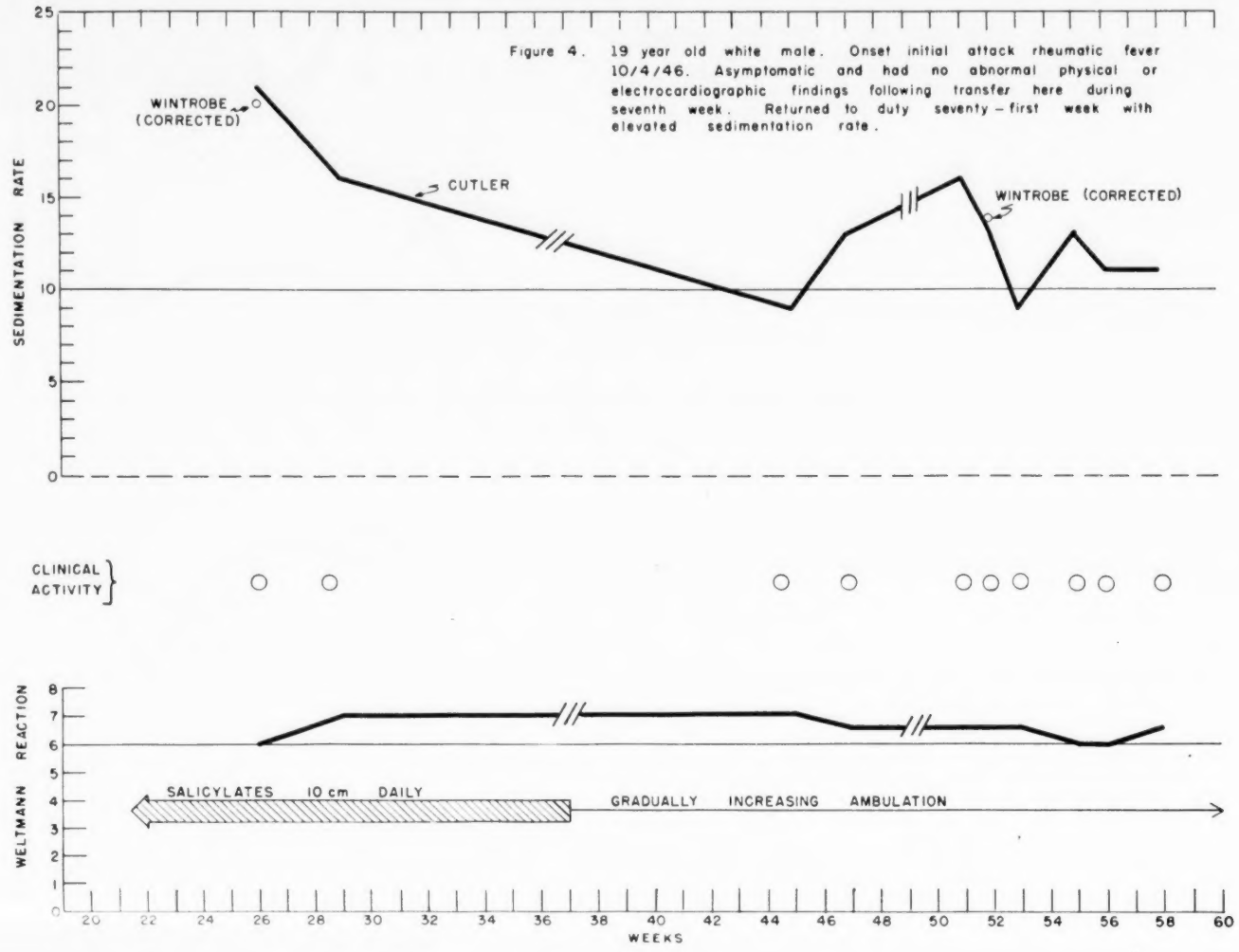


Figure 4. 19 year old white male. Onset initial attack rheumatic fever 10/4/46. Asymptomatic and had no abnormal physical or electrocardiographic findings following transfer here during seventh week. Returned to duty seventy – first week with elevated sedimentation rate.
SEDIMENTATION RATE
25
20
15
10
5
0
WINTROBE (CORRECTED)
CUTLER
WINTROBE (CORRECTED)
CLINICAL ACTIVITY
WELTMANN REACTION
8
7
6
5
4
3
2
1
0
SALICYLATES 10 cm DAILY
GRADUALLY INCREASING AMBULATION
20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50 52 54 56 58 60
WEEKS

Fig. 4.

entertained by several observers although serial blood cultures had been negative. During the thirty-second week the patient rather suddenly developed a rapidly progressive heart failure and died in spite of treatment. The post-mortem examination revealed an active myocarditis and endocarditis superimposed on an old aortic and mitral valvular disease, pericardial adhesions, and the usual evidences of passive congestion.

The laboratory data is tabulated below:

TIME FROM ONSET OF RHEUMATIC FEVER (WEEKS)	CUTLER SED. RATE (MM./HR.)	WELTMANN REACTION (TUBE)	CLINICAL ACTIVITY
20	29	4.0	+
23	32	3.0	+
24	30	4.5	+
25	35	4.0	+
26	30	5.5	+
27	27	3.5	+
28	28	4.5	+
29	21	4.5	+
30	24	5.5	+
31	29	4.0	+
32	23	5.0	+

This is one of the two patients included in the 400 under study who died of active rheumatic fever. Both the sedimentation rate and Weltmann reaction were in agreement with the clinical course and the pathologic findings. The Weltmann reaction provided strong evidence against the clinical impression of subacute bacterial endocarditis which was entertained because of this patient's failure to show a greater degree of clinical improvement and his prolonged febrile course. Although our experience has been limited and largely confined to suspected and later disproved cases, the Weltmann reaction has behaved as described by others[6] in differentiating active rheumatic carditis from subacute bacterial endocarditis by exhibiting a "shift to left" in the former as compared to a normal zone reaction in the latter condition. This has been explained as being due to the exudative reaction which accompanies active rheumatic carditis and the lack of exudation in subacute bacterial endocarditis.

SUMMARY

1. The Weltmann serocoagulation reaction proved to be a laboratory adjunct of considerable value in the prediction of rheumatic activity in a large group of naval patients hospitalized with rheumatic fever.

2. Of a total of 2,552 observations upon 400 patients with or convalescent from rheumatic fever, the Weltmann reaction correlated with the clinical status 97.2 per cent of the time against a correlation of 86.6 per cent for the Cutler sedimentation rate.

3. In 46 patients who exhibited rheumatic activity during the study, the Weltmann reaction correlated with the clinical status 93 per cent of the time against a Cutler sedimentation rate correlation of 90 per cent of the time during periods of activity. The respective correlations during periods of inactivity on these patients were 99 per cent for the Weltmann and 93 per cent for the sedimentation rate.

4. Of a total of 1,857 observations upon 350 patients who exhibited no rheumatic activity during the course of study, the Weltmann correlated 98.1 per cent of the time and the sedimentation rate 85.9 per cent of the time with the clinical observations. When the 22 patients who had four or more abnormal sedimentation rates were excluded, the Cutler sedimentation rate correlation was raised to 92.2 per cent.

5. Certain patients contributed more than their share of errors and were significantly important in the greater percentage of discrepancies which occurred in the sedimentation rate. These patients were those who failed to show expected increases of the sedimentation rate during periods of activity, and second, those who exhibited persistently elevated sedimentation rates following the cessation of activity. Similar persistent discrepancies between the Weltmann reaction and the clinical course of the patient were not observed.

6. The Weltmann reaction is of particular value as a complementary test to the sedimentation rate in the handling of large numbers of rheumatic patients where the physician must depend on laboratory evidences of rheumatic activity to a large extent in the management of patients under his care. It is also of particular value where discrepancies between the sedimentation rate and the clinical course leave him undecided as to the state of activity of the rheumatic process in his patient.

We deeply appreciate the aid given us by Captain R. L. Ware, M.C., U.S.N., Director, and Mr. W. V. Charter, Deputy Director, Medical Statistics Division of the Bureau of Medicine and Surgery, Navy Department, in the detailed statistical analysis of the data. We also appreciate the technical assistance of Chief Hospitalmen G. N. Stadler and G. H. Grimes, U.S.N., whose proficiency and devotion to duty allowed the accomplishment of the work involved in this study.

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THE INFLUENCE OF URINARY pH ON THE RENAL EXCRETION OF SALICYL DERIVATIVES DURING ASPIRIN THERAPY

WILLIAM S. HOFFMAN, M.D., PH.D., AND CATHERINE NOBE, B.S.
CHICAGO, ILL.

THE use of large doses of salicylates in the treatment of acute rheumatic fever has aroused increasing interest in the metabolism and excretion of this drug. Kapp and Coburn[1] have demonstrated that when sodium salicylate is administered either perorally or intravenously about 80 per cent of the drug is excreted in forms containing the salicyl radicle over a period of seventy-two hours. The remaining 20 per cent cannot be accounted for. Of the excreted salicyl compounds, about 20 per cent was recovered by these authors in the form of free salicylate, about 55 per cent as salicyluric acid (orthohydroxyhippuric acid), and most of the remaining 25 per cent as glycuronides. Such conjugated salicylates have not been found to be present in the plasma. In fact, after sodium salicylate administration, the salicyl compounds extractable from the plasma by ethylene chloride, chloroform, or other organic solvents are practically the same whether the extraction is or is not preceded by hydrolysis with strong acid.[2, 3, 4, 5]

That the rate of excretion of salicylate is dependent upon the pH of the urine has been suspected for some time, in spite of the statement of Goodman and Gilman[6] to the contrary. Morris and Graham,[7] as early as 1931, reported an increase of salicylate in the urine after bicarbonate administration. Smull, Wegria, and Leland[8] showed that bicarbonate simultaneously administered with salicylate produced a lower plasma salicylate level than when no bicarbonate was given. These authors surmised that the lowering was at least in part due to the increased renal excretion of salicylate. These findings have been confirmed by Smith and co-workers[4] and by Williams and Leonards.[9]

The most comprehensive study of the effect of alkali upon the excretion of salicylate was made by Smith and co-workers.[4] They developed an ingenious method for the quantitative partition of urinary salicylate fractions, based on the differential extractability of free salicylate, salicylurate, and salicyl glycuronides by ethylene dichloride and carbon tetrachloride and on the differential hydrolyzability of these fractions on boiling with acid. Smith and colleagues derived equations which allowed them to estimate total salicylate (ST), free salicylate (SA), and salicyluric acid (SU). By utilizing this method in the study of salicylate excretion in normal subjects and in patients with rheumatic fever, these authors demonstrated that the administration of sodium bicarbonate with salicylate lowered the plasma salicylate levels and that this lowering was produced by the increased excretion of free salicylate as the pH rose above 7.0.

From the Hektoen Institute for Medical Research of the Cook County Hospital, and from the Department of Physiology, University of Illinois College of Medicine.

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Smith's report appeared while we were engaged in a study of the biochemical changes involved in the development of salicylism during aspirin therapy of acute rheumatic fever and in acute aspirin poisoning. The increased excretion of salicylate on administration of bicarbonate had already become manifest from a study of the apparent salicylate clearances determined from the plasma salicylate concentration and from the extractable urinary salicylate. The method of Smith and associates gave us a more precise tool for the investigation of salicylate excretion. This report represents the results of these renewed studies. They are a corroboration and extension of the findings of Smith which have led to some new thoughts on the physiologic function of detoxication of salicylate by conjugation. Smith's studies were made on subjects receiving sodium salicylate; the present studies were carried out on convalescing rheumatic fever patients to whom aspirin was being administered as the therapeutic agent. A preliminary report of these studies already has been published as part of a clinical study of aspirin therapy in rheumatic fever.[10]

METHODS

Plasma salicylate determinations were carried out essentially by the method of Brodie, Udenfriend, and Coburn.[2] When low plasma concentrations were expected, the modification of Lester, Lolli, and Greenberg[3] was used.

For the partition of urinary salicylate the scheme of Smith and co-workers[4] was utilized. However, the equations were developed by an independent study of aqueous and urinary mixtures of sodium salicylate and salicyluric acid. Since our mathematical derivation of equations was attained by a somewhat different approach from that of Smith and since the resulting equations differ, if only slightly, from those of Smith, the scheme of development is offered here in detail.

Stock standard solutions of sodium salicylate and of salicyluric acid* were prepared, color value was equivalent only to 0.02 per cent of salicylate in the salicyluric acid, a value each equivalent to 100 mg. of salicylate per 100 c.c. (0.1160 Gm. sodium salicylate and 0.1410 Gm. of salicyluric acid per 100 c.c.) Calibration curves were made by photometric analysis of dilutions of these stock solutions with ethylene dichloride (EDC) and with carbon tetrachloride (CCl_4) as the extracting solvents. CCl_4 was found to extract the same percentage of salicylic acid from the acidified sodium salicylate solution as did EDC (over 90 per cent). These identical calibration curves were thereafter called the EDC sodium salicylate curves.

CCl_4 extracted practically no salicyluric acid from its aqueous solution. The average of the same order as the 0.04 per cent found by Smith. On the other hand, EDC extracted a considerable portion of salicyluric acid which gave a measurable color when made up with aqueous ferric nitrate solution. When the photometric readings for the various concentrations of the salicyluric acid obtained in making the salicyluric acid curve were read on the EDC sodium salicylate curve, as illustrated in Table I, it was found that an average of 52 per cent of the salicylurate present was analyzed by this procedure.

Now calibration curves were made for both sodium salicylate and for salicyluric acid with EDC extraction after hydrolysis for three hours with concentrated hydrochloric acid in a boiling water bath. The "hydrolyzed" sodium salicylate curve should have been identical with that obtained by analysis of unhydrolyzed samples extracted with EDC. Actually they were found to differ slightly, possibly because of the presence of strong acid and of traces of decomposition products. The "hydrolyzed" sodium salicylate curve was therefore retained as a separate curve for use with all hydrolyzed samples. Salicyluric acid when hydrolyzed gave a calibration curve different from that of nonhydrolyzed salicyluric acid because

*Crystalline salicyluric acid of high purity was made available to us through the courtesy of Dr. G. E. Cronheim of the S. E. Massengill Company, Bristol, Tenn. Nitrogen analysis of this sample showed 7.2 per cent nitrogen (theoretical 7.2 per cent).

TABLE I. THE PERCENTAGE OF SALICYLURIC ACID EXTRACTED BY ETHYLENE DICHLORIDE BEFORE AND AFTER HYDROLYSIS AS DETERMINED FROM SODIUM SALICYLATE CALIBRATION CURVE

A CONC. OF SALICYLURIC ACID IN SAMPLE (MG. PER 100 C.C.)	B PHOTOMETRIC READINGS ON EDC SALICYLURIC ACID CURVE (C, FIG. 1)	C VALUE OF READINGS IN COLUMN B ON EDC SODIUM SALICYLATE CURVE (A, FIG. 1) (MG. PER 100 C.C.)	D PER CENT OF SALICYLURIC ACID EXTRACTED BY EDC AS DETERMINED ON EDC SODIUM SALICYLATE CURVE (%)	E PHOTOMETRIC READINGS ON EDC HYDROLYZED SALICYLURIC ACID CURVE	F VALUE OF READINGS IN COLUMN E ON EDC "HYDROLYZED" SODIUM SALICYLATE CURVE (MG. PER 100 C.C.)	G PER CENT OF SALICYLURIC ACID EXTRACTED AFTER HYDROLYSIS AS READ ON EDC "HYDROLYZED" SODIUM SALICYLATE CURVE (%)
50.0	67.8	25.8	51.6	58.5	37.5	75.0
40.0	72.8	20.5	51.2	64.5	30.5	76.2
30.0	78.8	15.5	51.7	72.0	23.0	76.7
20.0	85.0	10.5	52.5	80.0	15.2	76.0
15.0	88.5	7.8	52.5	84.8	11.4	76.0
			Average 52.0*			Average 76.0

*When the photometric readings of Column B are read on the EDC hydrolyzed curve (*D*, Fig. 1), the values show an average per cent of extracted salicyluric acid of 55.0.

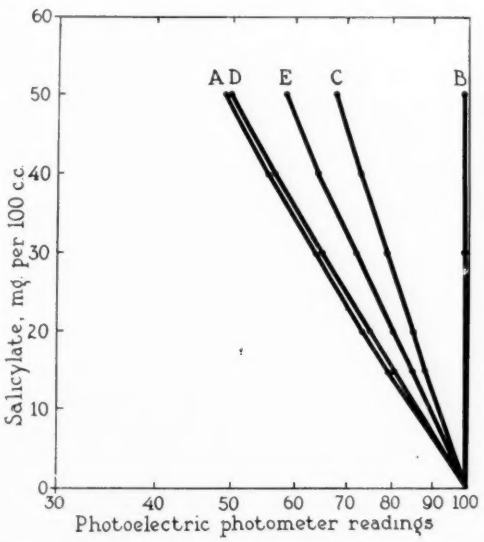

Fig. 1.—Photometric calibration curves for sodium salicylate and salicyluric acid. Curve *A* is for EDC sodium salicylate, *B* for CCl_4 salicyluric acid, *C* for EDC salicyluric acid, *D* for EDC (hydrolyzed) sodium salicylate, and *E* for EDC (hydrolyzed) salicyluric acid.

in three hours some, but not all, of the salicyluric acid had been hydrolyzed to salicylic acid. If the hydrolysis had been complete, the curve would have been identical with that of the "hydrolyzed" sodium salicylate curve. The actual photometric readings of the hydrolyzed salicyluric acid samples when read on the "hydrolyzed" sodium salicylate curve gave values that were 76 per cent of those obtained with corresponding amounts of sodium salicylate. But if the unhydrolyzed salicyluric acid when analyzed by EDC extraction had been read on the "hydrolyzed" sodium salicylate curve, the color values obtained would have been 55 per cent of the corresponding sodium salicylate quantities. Therefore, boiling for three

hours with acid had increased the color value from 55 per cent to 76 per cent. The actual percentage of hydrolysis of salicyluric acid was 76-55/100-55 × 100 or 46.7 per cent. These calculations are illustrated in Table I and the calibration curves are shown in Fig. 1.

One more observation is pertinent. Salicyl glycuronide, which is also found in urine after ingestion of salicylate, gives no color when analyzed by extraction with EDC or CCl_4. After hydrolysis by boiling with hydrochloric acid for three hours in a water bath, the glycuronide is completely converted to salicylic acid and gives 100 per cent of its expected color on the "hydrolyzed" sodium salicylate curve.

From these observations it is possible to calculate the concentrations of free salicylate (SA), salicyluric acid (SU), and salicyl glycuronide (SG) as well as the total salicyl compounds (ST) in any mixture of these compounds such as a urine sample. The calculations are made from equations obtained by the following algebraic reasoning.

When a mixture of SA, SU, and SG are analyzed by CCl_4 extraction and the photometric readings are read off on the EDC (or CCl_4) sodium salicylate curve, the analysis gives all of the SA, 2 per cent of the SU, and none of the SG. Therefore,

$$[CCl_4] = SA + 0.02\ SU \qquad \text{Equation 1.}$$

The same analysis made by EDC extraction and read on the same curve gives a value which represents all of the SA, 52 per cent of the SU, and none of the SG. Therefore,

$$[EDC] = SA + 0.52\ SU \qquad \text{Equation 2.}$$

If Equation 1 is subtracted from Equation 2, the following is obtained:

$$[EDC] - [CCl_4] = 0.50\ SU \text{ or} \qquad \text{Equation 3.}$$

$$SU = 2\ ([EDC] - [CCl_4])$$

If the mixture is hydrolyzed with hydrochloric acid and analyzed by EDC extraction, the reading on the "hydrolyzed" sodium salicylate curve represents all of the SA, 76 per cent of the SU, and all of the SG. Therefore,

$$[EDC(\text{hydrolyzed})] = SA + 0.76\ SU + SG \qquad \text{Equation 4.}$$

But by definition, total salicylate, ST, in the sample is characterized as follows:

$$ST = SA + SU + SG \qquad \text{Equation 5.}$$

Subtracting Equation 4 from Equation 5 gives

$$ST - [EDC(\text{hydrolyzed})] = 0.24\ SU \text{ or} \qquad \text{Equation 6.}$$

$$ST = [EDC(\text{hydrolyzed})] + 0.24\ SU$$

Thus by three analyses of the samples, [EDC], $[CCl_4]$, and [EDC(hydrolyzed)], it is possible to calculate SU from Equation 3, then SA from Equation 1, and ST from Equation 6. SG of course can then be calculated from Equation 5. These equations are essentially the same as those derived by Smith and co-workers, except that in their equation 6, the coefficient of SU was 0.33 instead of 0.24.

In an actual analysis of a urine sample, the procedure followed was that described by Smith. The urine was diluted 1 to 10 or 1 to 20, and an aliquot of 1 c.c., acidified with 0.5 c.c. of 6N HCl, was extracted with 30 c.c. of EDC or CCl_4 by shaking for five minutes. The extract was then treated with 10 c.c. of water and 0.25 c.c. of a 1 per cent ferric nitrate solution in 0.07N nitric acid and again shaken for five minutes. The colored aqueous solution was removed and read in a photoelectric photometer* with water set at 100 and a green filter with maximal transmission at 530 mμ. The reading of the EDC analysis on the EDC sodium salicylate curve in milligrams salicylate per 100 c.c. is the [EDC] value, while that for the CCl_4 analysis on the same curve is the $[CCl_4]$ value. For the EDC analysis of the hydrolyzed sample, 0.5 c.c. of the diluted urine specimen was treated with 1 c.c. of concentrated hydrochloric acid and heated in a boiling water bath for three hours, cooled, diluted

*The Cenco Photelometer was used in these studies.

with 1 c.c. of water, and extracted with 30 c.c. of EDC. Then the color was developed by shaking with 10 c.c. of water and 0.25 c.c. of the ferric nitrate reagent, and the optical transmission was determined in the photoelectric photometer as before. The transmission read on the EDC "hydrolyzed" sodium salicylate curve gave the [EDC(hydrolyzed)] value.

The accuracy of the scheme of analysis is illustrated in the examples of analysis of known mixtures shown in Table II.

TABLE II. THE PARTITION ANALYSIS OF SALICYLIC AND SALICYLURIC ACIDS IN KNOWN MIXTURES BY MEANS OF SMITH SCHEME

		MILLIGRAMS PER 100 C.C.					
		SODIUM SALICYLATE		SALICYLURIC ACID		TOTAL SALICYLATE	
NO.	SAMPLE	PRESENT	FOUND	PRESENT	FOUND	PRESENT	FOUND
1	Water	10.0	9.90	30.0	31.6	40.0	40.3
2	Water	20.0	20.1	10.0	9.4	30.0	30.1
3	Water	15.0	14.5	15.0	15.0	30.0	29.6
4	Urine	10.0	9.6	90.0	86.6	100.0	105.8

Other analyses utilized in this study were serum and urinary chloride by the method of Schales and Schales[11] and creatinine by a photoelectric adaptation of the Folin method.[12]

The experiments which constitute the basis of this report were clearance studies carried out on rheumatic fever patients who were in the convalescent stage but who were still receiving from 6 to 9.6 Gm. of aspirin daily in divided doses at six-hour intervals. The first clearances were performed under the usual conditions of therapy in the morning about two hours after the first morning dose of aspirin. One hour before the test, the patient was asked to drink 500 c.c. of water. The urine was collected uncatheterized in two one-hour periods, and blood specimens were drawn at 0, 1, and 2 hours. The mean of the first two plasma salicylate levels was used as the average plasma concentration for the first period, and the mean of the second and third for the second period. Creatinine and chloride determinations were made on all plasma and urine specimens. The subjects were then given sodium bicarbonate in addition to the dose of aspirin previously administered (2.0 to 3.3 Gm. of sodium bicarbonate every six hours). At the end of three days, the clearance experiments were repeated after the morning dose of aspirin and sodium bicarbonate.

Clearance studies were made in sixteen subjects, but only in thirteen of these were analyses of the urinary salicylate fractions successfully executed. In two subjects a peculiar brown discoloration occurred on the addition of the iron reagent to the extracted urinary salicylate which made accurate analysis impossible. Whether this discoloration was due to some extraneous metabolite or medicament or whether it was produced by one of the rare breakdown products of salicylates found occasionally in the urine by Kapp and Coburn[1] could not be determined. In one other experiment, the CCl_4 extraction for some unknown reason gave higher values than the EDC extraction, which phenomenon also precluded calculation of the urinary salicylate fractions. In the other thirteen experiments, clearance estimations could be made satisfactorily and the ratios of the salicylate fractions, though variable, showed a consistent pattern.

RESULTS

Plasma Salicylate Levels and Their Relation to Urinary pH.—Since in all of these experiments each patient was on a constant intake of aspirin which already had been demonstrated to keep the plasma salicylate concentration nearly constant, any pronounced change in this level on the addition of sodium bicarbonate to the regimen would very likely reflect the influence of the sodium bicarbonate, especially if the change was consistent. Fig. 2 shows the change in plasma salicylate in nineteen subjects after three days of sodium bicarbonate medication. In all instances there was a significant fall in the plasma salicylate

levels, at times as much as 20 mg. per 100 cubic centimeters. The fall was roughly proportional to the accompanying increase in urinary pH. These findings are in corroboration of those reported by other recent investigators.[3, 4, 8, 9] The mechanism of this drop in salicylate concentration and its significance will be discussed further later.

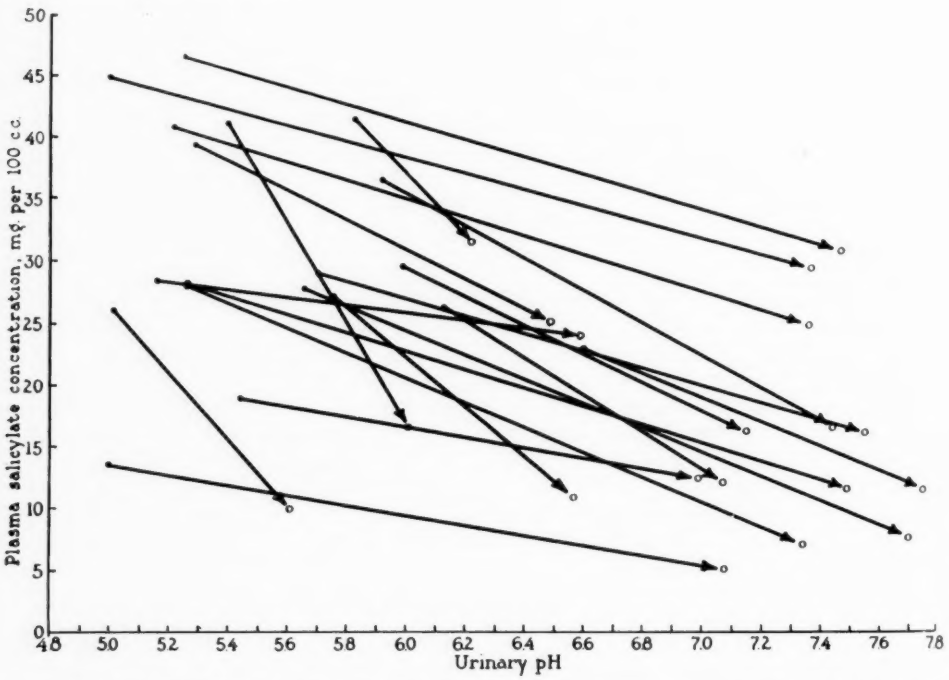

Fig. 2.—The fall in plasma salicylate levels after sodium bicarbonate administration for three days in nineteen subjects. Each arrowed line shows the original (solid circle) and final (hollow circle) plasma salicylate concentration in relation to the urinary pH for each subject.

The Clearance of Salicylate.—In Table III the clearances of unconjugated salicylate (SA) and of total salicylate are shown in Columns 5 and 6. The clearances and the urinary pH for each period are the means of two determinations. These clearances have been expressed as ratios of the salicylate clearance to the creatinine clearance in order to rule out the effect of lowered glomerular filtration rate which was found to be produced by the alkalinizing regimen. Such a procedure also rules out potential errors due to incomplete collections and allows comparison of the clearances of the various subjects in spite of any incidental diminution in renal function associated with the rheumatic fever or the rheumatic carditis. Actually the salicylate clearances calculated from the plasma salicylate levels are only a fraction of the true clearance, for only a portion of the plasma salicylate is in the unbound state and filterable through the glomeruli. The remainder is bound to plasma proteins. An estimation of the unbound plasma salicylate fraction has been made by several workers[3, 4, 9] by analyzing ultrafiltrates of plasma specimens containing salicylate. Smith and associates,[4] for example, found about 20 to 25 per cent of the plasma salicylate to be in the

TABLE III. THE CLEARANCES OF CHLORIDE, FREE SALICYLATE (SA), AND TOTAL SALICYLATE (ST) DURING ASPIRIN THERAPY WITHOUT AND WITH ADDED SODIUM BICARBONATE

CASE	SODIUM BICARBONATE (GM. PER DAY)	URINE pH	cl/c × 100	SA/C × 100	ST/C × 100
1	0	5.99	1.73	1.99	4.48
	8.0	7.14	5.66	1.75	44.0
2	0	5.83	1.93	4.06	10.20
	8.0	6.22	1.71	4.17	11.26
3	0	5.44	1.51	0.50	12.35
	13.3	6.99	2.94	1.80	24.65
4	0	5.92	2.23	3.98	11.26
	13.3	7.43	1.80	23.25	41.5
5	0	6.60	3.16	9.66	18.15
	13.3	7.75	3.92	26.5	38.4
6	0	5.70	.98	2.56	12.8
	13.3	7.55	1.23	24.35	38.3
7	0	5.26	3.06	1.80	9.22
	13.3	7.49	1.76	20.33	32.84
8	0	5.41	1.53	1.76	13.78
	13.3	6.08	1.39	5.13	42.1
9	0	5.13	1.53	0.70	6.80
	13.3	7.42	1.55	9.99	20.53
10	0	5.04	3.17	1.26	12.69
	5.3	5.61	2.99	4.77	31.37
11	0	5.29	1.67	1.07	14.1
	8.0	6.48	.68	11.02	25.52
12	0	6.85	1.03	1.31	12.65
	13.3	7.62	1.11	16.33	32.08
13	0	6.13	.90	0.60	7.18
	13.3	7.04	1.17	6.64	17.10

Cl/C, Chloride clearance ÷ creatinine clearance.

SA/C, Ratio of salicylate clearance to creatinine clearance, when the salicylate clearance is estimated from urinary free salicylate (SA) and plasma salicylate with no account taken of the bound portion in the plasma.

ST/C, Same as SA/C except that urinary total salicylate (ST) is utilized.

unbound form at levels of the order of 30 mg. per 100 cubic centimeters. If these values are correct, the true clearances (and the ratios to the creatinine clearances) are four to five times greater than the apparent clearances reported here. However; since it has been shown by Williams and Leonards[9] that alkalinization produces no change in the percentage of salicylate bound to protein, the effect of bicarbonate on the excretion of salicylate can be ascertained with a fair degree of accuracy from the apparent clearances.

In the thirteen control experiments, when aspirin was being administered without sodium bicarbonate, the clearances of free unconjugated salicylate (SA) ranged from 0.50 to 9.66 per cent (Column 5, Table III). However, nine of the thirteen values were below 2.0. The median value was 1.76. These clearance values were obtained when the urinary pH was in the acid range, from 5.04 to 6.85. It will be noticed that the values are of the same order as the chloride clearances (Column 4, Table III), also expressed as ratios of the creatinine clearance. Since chloride clearances are normally low because most

of the chloride of the glomerular filtrate is reabsorbed with sodium and water as an isotonic solution to form the so-called obligatory reabsorption, the correspondingly low SA clearance is very likely due to the same phenomenon.

In each subject the addition of sodium bicarbonate to the medication produced a considerable increase in the SA clearances. With one exception, the values were increased three to thirteen times. In the exceptional instance (Case 2), the daily intake of 8.0 Gm. of bicarbonate produced only a slight rise in urinary pH, and the SA clearance was correspondingly little affected. In Cases 4, 5, 6, and 7, where the urines ranged from 7.43 to 7.75, the new SA clearances were more than 20 per cent of the creatinine clearance. If the apparent clearance was only a fourth or a fifth of the true clearance, these findings would signify that when the urine was made strongly alkaline, free salicylate was excreted by the kidney almost as well as was creatinine, mannitol, or inulin.

Chloride clearances did not show a corresponding increase on alkalinization of the urine. With the exception of Case 1, in which a substantial rise in chloride clearance took place, the changes in the other experiments were equivocal in both directions. If the presence of an excess of sodium prevented the reabsorption of salicylate ions from the glomerular filtrate, it did not under the conditions of these experiments accomplish the same thing for chloride, the reabsorption of which is apparently dependent upon many other factors.

In Column 6, Table III are shown the clearances of total salicylate, expressed as ratios of the creatinine clearance. From a physiologic point of view the total salicylate clearance does not represent a single functional entity. It expresses the excretion of at least three forms of salicylate in the urine on the basis of concentrations of salicylate in plasma which is known to be only of one form, the unconjugated form (SA). Nevertheless the total salicylate clearance can be utilized as a means of comparing the over-all ability of the kidneys to remove salicylate from the blood for any given level of plasma salicylate in the two regimens with and without sodium bicarbonate. The data show a significant increase in the clearance in each instance. With the exception of Case 2, the clearances were increased two to three times by the administration of bicarbonate. The highest value found was 44 per cent of the creatinine clearance. Such a value, when multiplied by four or five to give the true clearance on the basis of the filterable plasma salicylate, would give a clearance about two times the amount of the creatinine clearance.

In Fig. 3, the SA and ST clearances are plotted against the urinary pH values for all thirteen experiments. Though the points show considerable scattering, it is easily recognizable that the SA clearance is directly related to the urinary pH. The higher the pH, the greater the clearance of free salicylate. This relationship is more pronounced as the pH rises above 7.0. The same type of relationship can be seen for the total salicylate. In the acid range, the ST clearance values lie bunched between 6 and 16, whereas in the alkaline range the values spread from 20 to 44.

The Relation of the Partition of Urinary Salicylate to the Urinary pH.—In Fig. 4 the ratios SA/ST, SU/ST, and SG/ST are plotted against the urinary

pH for all urine samples analyzed by the Smith scheme. There is obviously a great degree of scattering but a pattern is discernible. The chief effect of increase in alkalinity in the urine is a marked rise in the SA/ST ratio. Whereas in strongly acid urine, SA represents only 10 to 20 per cent of the total excreted salicylate (quite as was found by Kapp and Coburn[1] by direct isolation

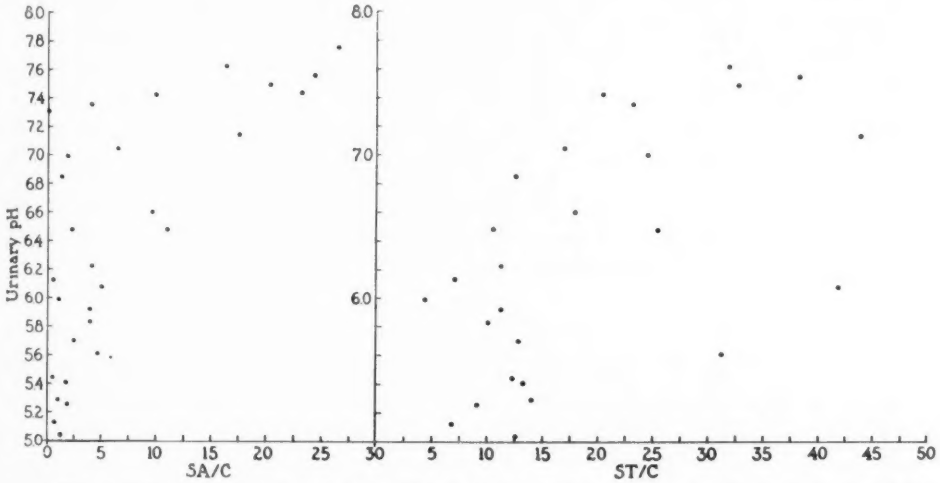

Fig. 3.—The relation of the clearance of free salicylate (SA/C) and of total salicylate (ST/C) to the urinary pH. Clearances are expressed as percentages of the corresponding creatinine clearances.

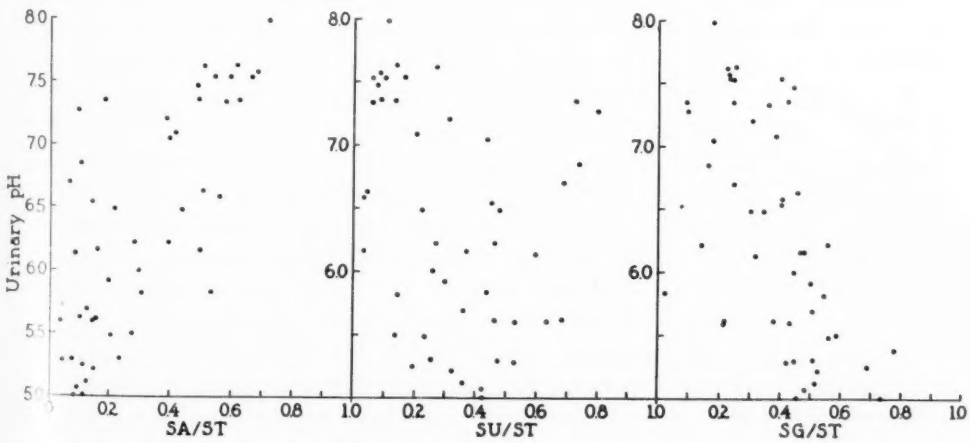

Fig. 4.—The relation of the distribution of urinary salicylate fractions to the urinary pH.

methods), in the alkaline urine that percentage may rise to 70. In the eight experiments in which the administration of sodium bicarbonate led to a definitely alkaline urine, the average SA/ST ratio was found to change from 0.194 to 0.519.

The effect of alkalinization on the excretion of salicyluric acid and salicyl glucuronide could not be determined from these clearance studies. In the eight

experiments just mentioned, the average SU/ST ratio was 0.348 and the average SG/ST was 0.456. Kapp and Coburn found a larger proportion of salicyluric acid, as was indeed present in several of our cases. It is possible that SG/ST is larger in the present studies because SG represents in addition to salicyl glycuronide any unchanged acetylsalicylic acid that reached the blood stream and was excreted unchanged. However, it is unlikely that such an excretion is of any considerable magnitude. With the large rise in the SA/ST ratio accompanying alkalinization, the SU/ST and SG/ST ratios naturally fall—this tendency can be recognized in Fig. 4—but whether there is an absolute change in either or both excretions is not clear. Nor is it easily discernible from Fig. 4 or from the changed ratios in each individual experiment whether the increased excretion of SA is more at the expense of SU or of SG. In most instances SU/ST and SG/ST ratios dropped parallelly. The individual ratio data have been omitted, since they offer no more information than can be obtained from Fig. 4.

DISCUSSION

Analysis of the findings presented makes certain facts clear. In the first place it can be seen that under the normal conditions of an acid urine relatively little of the excreted salicylate is in the form of free salicylate ions. About 80 per cent is excreted in the form of conjugated salicylate. These proportions have been found both for sodium salicylate administration by Smith and coworkers[4] and for aspirin by us. The clearance of free salicylate ions, even if calculated on the basis of the concentration of unbound salicylate in the plasma, is of a low order, not much greater than that of chloride. It must be inferred then that in acid urines salicylate ions, like chloride, are for the most part reabsorbed from the glomerular filtrate. If this were the only means of excretion of salicylate, aspirin administration would produce much higher plasma salicylate levels than it actually does. It is through the excretion of the conjugated forms of salicylate under these circumstances that the body succeeds in getting rid of salicylate as well as it does. Even with this aid, the excretion of salicylate is slow. Single doses of aspirin, for example, have been found by Lester and associates[3] to require thirty or more hours for complete excretion. Multiple large doses of aspirin spread at four-hour intervals produce a pyramiding rise in the plasma salicylate level which can soon lead to severe intoxication.

When alkali is administered along with aspirin, the excretion of salicylate ions becomes markedly improved. This increased excretion is demonstrable not only from the rise in SA clearances, but also from the elevation of the SA/ST ratios. The extent of this improvement in SA excretion is great enough to increase the total excretion of salicylate by 100 to 200 per cent, and thereby to produce a marked fall in the plasma salicylate concentrations. When the urine is alkalinized to a pH of 7.5 or higher, the clearance of SA, if calculated from the concentration of freely filterable salicylate in the plasma, becomes almost as great as that of inulin or creatinine. In other words, in the presence of enough excess sodium ions in the glomerular filtrate, it is possible to prevent the reabsorption of almost all the salicylate ions in that filtrate.

Lester and co-workers[3] have found the total salicylate clearances to be of the same magnitude as the urea clearances and they believe the manner of excretion of both to be the same. This conclusion is unwarranted even if the method for the total salicylate used by these workers is accurate. For actually total salicylate clearance is not a simple physiologic entity. It represents the total ability of the kidneys to remove salicylate from the blood by various channels. Only the portion of the total salicylate clearance which is that of free salicylate is clearly that of glomerular filtration and reabsorption. The clearance of conjugated salicylate will on analysis be recognized to be of a different type. The concentration of conjugated salicylate in the plasma has always been found to be zero or so close to zero as to be within the limit of error of the determination. At the most the concentration was less than 0.5 mg. per 100 cubic centimeters. On the other hand, the concentration of conjugated salicylate in the urine was often as high as 150 mg. per 100 c.c. when the urine output was around 2 c.c. per minute. If the plasma concentration of conjugated salicylate is truly zero, then the clearance of conjugated salicylate is infinitely high. Even if traces of both salicyluric acid and of salicyl glycuronide are present in the plasma—for example 0.2 or 0.3 mg. per 100 c.c. when the urine concentration is 100 mg. per 100 c.c.—the resultant clearances would be in the range of that of Diodrast or *p*-aminohippurate. In either case, it must be concluded that salicyluric acid and salicyl glycuronide are excreted predominantly by the tubules, either by virtue of being produced in the tubules and then secreted by them or by being removed from the peritubular blood by tubular excretion. From the known fact that both the liver and kidneys can make glycuronides and hippuric acids, it is reasonable to believe that the excretion is a combination of both tubular processes.

Conjugation has long been recognized as a means of detoxication by the body, of conversion of a potentially toxic substance into a less toxic one. In connection with salicylate, detoxication must be regarded as having a different meaning. Here it is the conversion of a potentially toxic substance, which under normal metabolic conditions the body is poorly equipped to excrete, into substances which can be rapidly excreted under any metabolic conditions.

SUMMARY

The validity of the scheme of Smith and associates for the partition analysis of the salicylate fractions in urine has been confirmed. The equations obtained differed only slightly from those of these workers.

This analytical method has been utilized to study the effect of sodium bicarbonate administration on the excretion of salicylate during aspirin therapy for acute rheumatic fever.

The apparent clearance of free salicylate during regimens of aspirin without added sodium bicarbonate ranged from 0.5 to 9.66 per cent of the corresponding creatinine clearances, but most of the values were below 2 per cent. The true clearances, based on the unbound plasma salicylate concentration, were probably four to five times as high. Even such clearances were of the order of

those of chloride, and this implied that the free salicylate filtered through the glomeruli was for the most part reabsorbed by the tubules.

The free salicylate constituted about 20 per cent of the total salicylate in the acid urines, the remaining 80 per cent being almost equally divided between salicyluric acid and salicyl glycuronide.

When sodium bicarbonate was administered along with the aspirin in quantities sufficient to alkalinize the urine, the free salicylate clearances were increased to three to thirteen times the original values. The clearances were found to be proportional to the urinary pH. This increased excretion of free salicylate made it comprise as much as 70 per cent of the urinary total salicylate, the average being 52 per cent. The total salicylate clearance was thereby also increased as much as 200 per cent. These clearances were also found to be proportional to the urinary pH. The enhanced clearances of total salicylate accounted for the significant drop in plasma salicylate levels, which occurred in all instances.

The true clearance of free salicylate in alkaline urines approached that of creatinine. Thus, excess of base prevented almost completely the reabsorption of free salicylate from the glomerular filtrate.

Since at the most only traces of conjugated salicylates were present in the plasma, the clearance of these conjugates was either infinitely high or at least as high as *p*-aminohippurate. Such findings indicated that conjugated salicylates either were produced in the renal tubules or excreted through them, or both. Conjugation of salicylate has for its function a special type of detoxication which converts the ordinarily poorly excretable free salicylate into compounds which are easily excretable.

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THE COMPARATIVE EFFECTS OF WATER, CARBONATED WATER, SODIUM CHLORIDE SOLUTION, AND SODIUM BICARBONATE SOLUTION ON GASTRIC ACID SECRETION IN HUMAN SUBJECTS

EDWARD H. HALE, M.D., A. C. IVY, M.D., PH.D., AND
M. I. GROSSMAN, M.D., PH.D.
CHICAGO, ILL.

FROM time to time one sees or hears the statement that carbonated water or sodium bicarbonate stimulates gastric secretion in the human being. The experiments which have been done[1] with sodium bicarbonate alone (i.e. with no other stimuli for gastric secretion) are too few in number and too variable to permit interpretation. No experiments with carbonated water in human subjects could be found.

This study was performed to determine whether carbonated water or sodium bicarbonate had a significantly different effect from an equal quantity of plain water or sodium chloride solution on the secretion of acid by the stomach of human subjects. Observations were also made on the rate of the gastric evacuation of these solutions from the stomach.

METHODS

The subjects were normal young adult males and, in those tests so designated, male adults in whom the diagnosis of duodenal ulcer had been established radiologically. One group of subjects were used for tests with distilled and carbonated water and a second group for the test with sodium chloride and sodium bicarbonate solutions. After an overnight fast, a stomach tube was passed and the gastric residuum was removed. At fifteen minute intervals basal gastric secretion was collected for three periods. The test solution was then introduced into the stomach through the stomach tube. After fifteen minutes the stomach was emptied, the volume measured, a 10 c.c. portion retained for titration, and the remainder returned to the stomach. The stomach was emptied of the remaining test solution at the end of the next fifteen-minute period and then samples were taken every fifteen minutes for one to one and one-half hours. In the tests with distilled and carbonated water, the same procedure was followed with the exception that ten-minute periods were used. All samples were titrated for acid content using Töpfer's reagent and phenolphthalein as indicators. The test solutions used were 250 c.c. volumes of (a) distilled water, (b) carbonated distilled water (containing 4.4 volumes of CO_2 per unit volume of water at 60° F., thus 250 c.c. contains as much CO_2 as can be released from 5.5 Gm. of $NaHCO_3$), (c) 0.9 per cent sodium chloride solution, and (d) 1.3 per cent (isotonic) sodium bicarbonate solution.

RESULTS AND DISCUSSION

The results are presented in Figs. 1, 2, and 3.

Only normal subjects were tested with distilled water and carbonated distilled water. No significant difference in rate of gastric emptying or in output of HCl evoked occurred with these two test solutions. The degree of stimulation of acid secretion was in keeping with the results of previous studies on water.[2]

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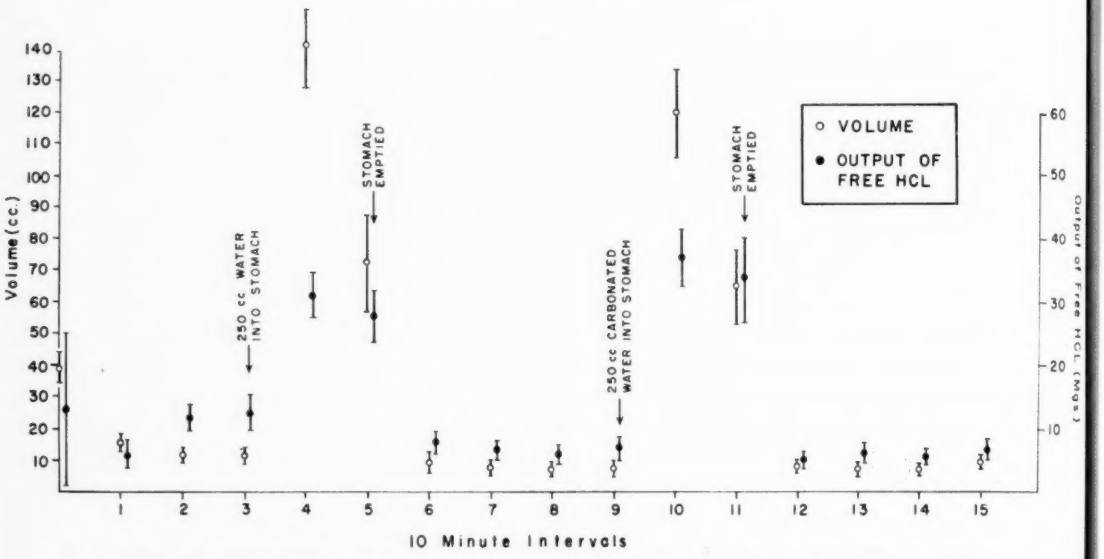

Fig. 1.—Comparison of volume of gastric contents and output of free HCl in response to distilled water and carbonated water. Each point represents the mean value of sixteen tests, two on each of eight subjects. The first set of points represents the gastric residuum at the beginning of the tests. The vertical lines extending from the points indicate the standard error of the mean.

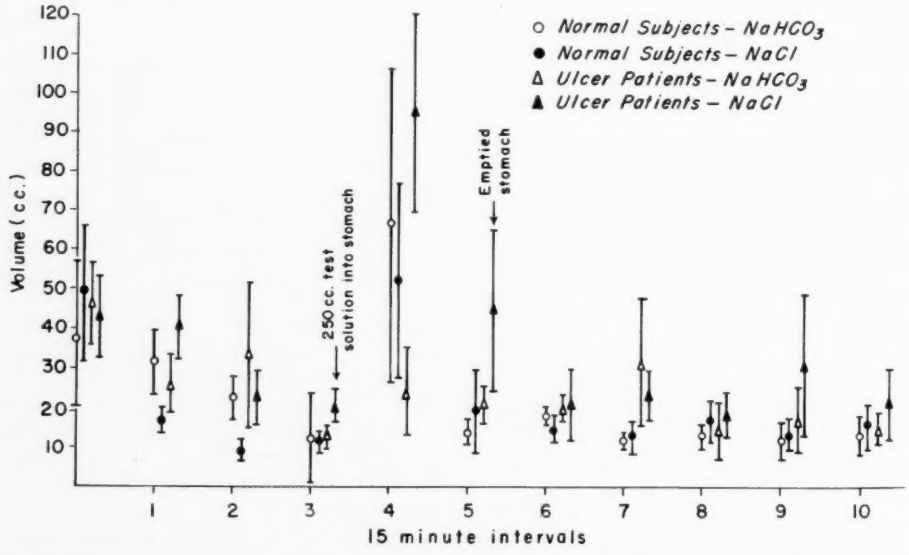

Fig. 2.—Volume response to NaCl and $NaHCO_3$. Each point is the mean value for five subjects. The lines extending from the points represent the standard error of the mean.

In normal subjects no significant difference between isotonic sodium chloride and isotonic sodium bicarbonate occurred in regard to rate of evacuation. In ulcer patients sodium bicarbonate solution left the stomach more rapidly than sodium chloride solution (statistically significant). Because of the high neutralizing capacity of the sodium bicarbonate solution it is not possible to detect

whether stimulation of acid secretion occurred during the time the solution was in the stomach. No stimulation was seen in the periods following the removal of the sodium bicarbonate. In other words there was no evidence of acid "rebound."

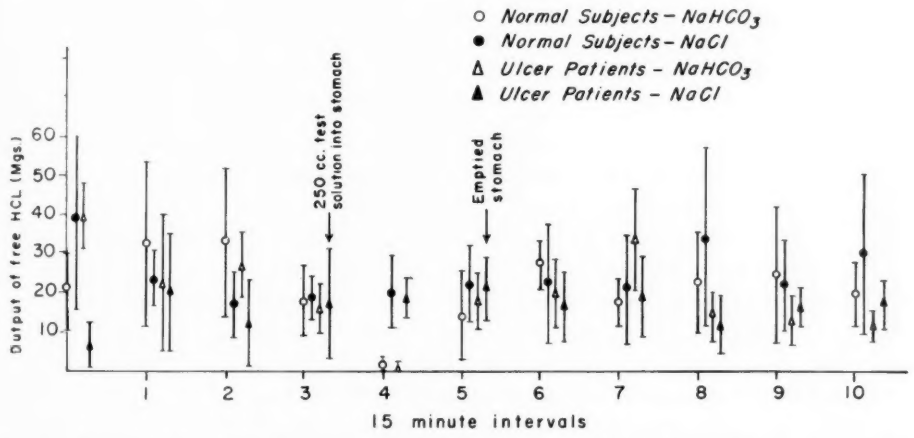

Fig. 3.—Output of free HCl in response to NaCl and $NaHCO_3$. These are the same tests for which volume values are given in Fig. 2. See legend of Fig. 2 for explana ion.

In contrast to water and carbonated water, isotonic saline solution did not stimulate acid secretion.

Crider and Thomas[3] have observed a similar difference between distilled water and isotonic saline solution in regard to stimulation of pancreatic secretion by introduction into the duodenum.

A satisfactory explanation of the mechanism of the stimulatory action of water on gastric secretion has never been given.[2] The possibility that water causes gastrin to be released should be considered.

SUMMARY

1. In normal human subjects: (a) carbonated water does not stimulate gastric secretion or affect gastric emptying to any greater extent than non-carbonated water; (b) isotonic sodium chloride and isotonic sodium bicarbonate solutions leave the stomach at about the same rate.

2. In duodenal ulcer patients isotonic sodium bicarbonate solution leaves the stomach more rapidly than isotonic sodium chloride solution.

3. In contrast to distilled water, isotonic sodium chloride solution does not stimulate gastric acid secretion.

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A CLINICAL STUDY OF THE USE OF NITROGEN MUSTARD THERAPY IN POLYCYTHEMIA VERA

CHARLES L. SPURR, M.D.,* TAYLOR R. SMITH, M.D., MATTHEW BLOCK, M.D.,† AND LEON O. JACOBSON, M.D.
CHICAGO, ILL.

CLINICAL investigation of the efficacy of nitrogen mustard, methyl-*bis*(β-chloroethyl) amine hydrochloride, in controlling polycythemia vera is warranted by its suppressive property upon hematopoietic tissue. A preliminary report of our experience in three cases of polycythemia vera was published in 1946.[2, 3] More recently others have commented upon their experience.[4, 5]

The influence of this compound upon hemopoiesis was followed in clinical studies of its use in the control of lymphomas.[6-9] In a survey of the influence of various doses upon the peripheral blood picture, Spurr and co-workers[8] showed that the standard course of four daily injections of 0.1 mg. per kilogram body weight reduced the erythrocyte and hemoglobin values over a period of six weeks following injection. Serial marrow aspirations have demonstrated that the regeneration of polychromatophilic and orthochromatic erythroblasts is not striking until the sixth week after therapy. This erythropoietic suppression appears to be temporary, with complete regeneration occurring even after many courses of the compound.[1, 8]

METHOD

A group of ten patients with polycythemia vera was studied. Their disease was of two to twenty years' duration—an average duration of seven years—and each had received several forms of therapy earlier in the disease. This prior therapy may serve as a comparative control period in considering the efficacy of nitrogen mustard. In preparation for nitrogen mustard therapy, repeated phlebotomies were carried out in seven instances to reduce the hematocrit to 60 per cent. Thus the risk of thrombosis during the early phase of treatment was reduced. Blood studies which included the determination of erythrocytes, hemoglobin by a photoelectric method (16.2 Gm. = 100 per cent), hematocrit,‡ reticulocytes using the moist chamber technique, leucocytes, and differential counts were repeated three times weekly in the control period, weekly in the first six weeks following therapy, and later at two- to four-week intervals, depending upon the clinical status of the patient. Each remission was considered to be concluded when the erythrocyte, hemoglobin, and hematocrit values increased above normal levels, the most reliable guide being the hematocrit which was considered elevated when it reached a level of 55 per cent.

The dose and the method of administration were identical to those used in the treatment of lymphomas.[2] An intravenous dose of methyl-*bis*(β-chloroethyl) amine hydrochloride equivalent to 0.1 mg. per kilogram body weight repeated on four consecutive days was used in each case. The immediate systemic toxic manifestations of nausea and vomiting were treated by sedation.

From the Department of Medicine, The University of Chicago.

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*Present address: Baylor University, College of Medicine, Houston, Texas.
†Senior Research Fellow, United States Public Health Service.

‡Wintrobe method.

RESULTS

A group of ten cases was observed over periods of from twelve to forty months. Each patient was in an active regenerative phase of the disease at the time of treatment. Table I presents the age, sex, duration of disease prior to this study, previous therapy, total dose of nitrogen mustard, the pretreatment and post-treatment blood counts, the interval to the lowest post-treatment hematocrit, and the total duration of remission. The exact remission from other forms of therapy is included in instances where this was known.

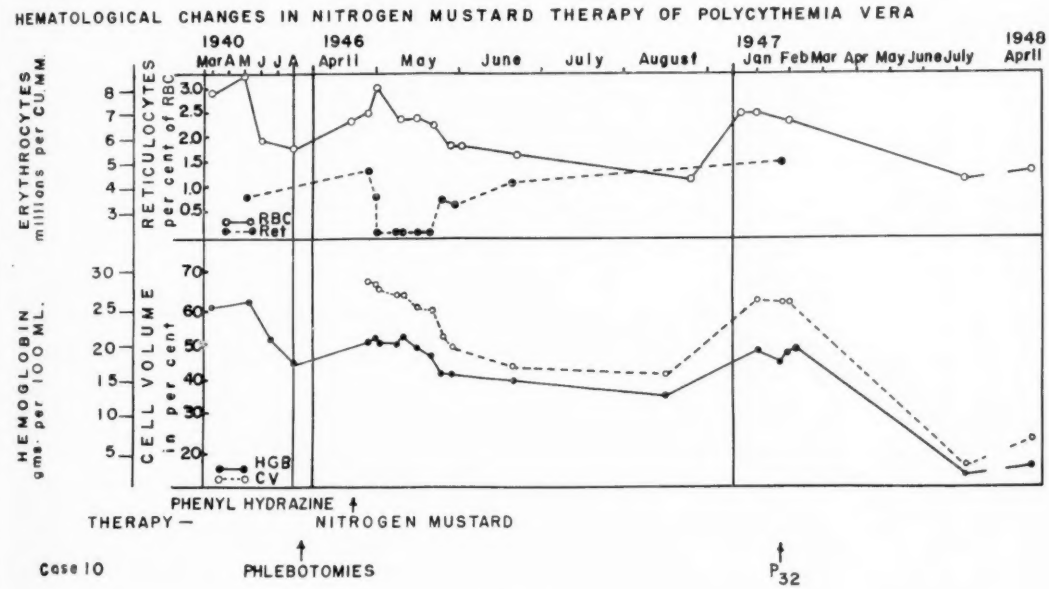

Fig. 1.

These ten patients received fifteen courses of nitrogen mustard. The average pretreatment hematocrit was 59 per cent, and the average minimum post-treatment hematocrit was 44 per cent. Utilizing the attainment of a hematocrit of 55 per cent as evidence of termination of remission, the nitrogen mustard produced an average remission of 6.8 months. The range, however, varied from two to thirty months. In eight instances the remission was five months or longer; in two, remission of three months or less resulted.

Leucopenia followed each course of the drug, but only in six patients, or 40 per cent, did the blood count fall below 3,000; in one half of these, below 1,500. The period of leucopenia was very brief and was not accompanied by complications; hence, no special therapeutic measures were required. The suppression of erythropoiesis by this dose of nitrogen mustard was indicated by the decline in the hematocrit. This occurred in an average of 4.4 weeks, with a range from four to ten weeks. The changes in the constituents of the peripheral blood picture in Case 10 are graphed (Fig. 1). In this patient the reticulocytes were suppressed to 0.5 per cent for three weeks following therapy and regained their

TABLE I. TEN PATIENTS WITH POLYCYTHEMIA VERA TREATED WITH NITROGEN MUSTARD; SUMMARY OF PERTINENT CLINICAL DATA

CASE AND INITIAL DATE	THERAPY	DOSE OF NITROGEN MUSTARD (MG.)	PRETREATMENT				INTERVAL AFTER TREATMENT TO LOWEST HCT.	BLOOD VALUES AT TIME OF LOWEST HCT.				TOTAL REMISSION IN MONTHS
			HGB.	R.B.C.	HCT.	W.B.C.		HGB.	R.B.C.	HCT.	W.B.C.	
1. 38 M	No. 339424; Duration—7 yr.											
8/3/44	Phlebotomy 500 c.c.		20	7.3	60	18.2		15.5	6.2	47	12.0	.75
1/3/45	Nitrogen mustard	34	19.4	7.6	62	9.6		14.4	4.6	42	8.6	-6*
8/21/45	Nitrogen mustard	34	17.5	5.9	51	14.0		15.4	4.9	43	7.2	5
2. 60 F	No. 351087; Duration—2 yr.											
1943	Phenylhydrazine—(acute hemolysis)											2
2/45	Phlebotomies		23.0	6.9	71	9.7		15.3	5.6	59	7.6	1
9/4/46	Nitrogen mustard—Phlebotomy	25.4	16.8	6.1	61	7.6	8 wk.	14.2	5.2	42	4.5	4
	Nitrogen mustard	19.5	18.3	5.7	58		6 wk.	16.2	4.8	45	4.5	5
3. 60 M	No. 367450; Duration—5 yr.											
10/9/45	P^{32}—13 mc. orally		16.2	6.5	61	26.0	No decline	18.4	8.7	72	12.0	0
1/20/46	Nitrogen mustard	28	21.0	10.4	75	28.0	3 wk.	15.8	7.1	60	1.3	2
	Nitrogen mustard (Phlebotomy 2 × 500 c.c.)	28	18.9	5.9	65	5.8	3 wk.	18.0	6.1	61		2.0
7/5/46	Nitrogen mustard (Phlebotomy 2 × 500 c.c.)	28	20.0	6.4	66	12.0	3 wk.	18.9	6.5	65	14.0	2.5
11/3/46	P^{32}—7 mc. I.V. (Phlebotomy 3 × 500 c.c.)		17.0	7.8	58	25.0		18.0	7.1	64	26.0	0†
4. 46 M	No. 316809; Duration—5 yr.											
9/43-5/44	Phlebotomies—3 mo.											
5/31-7/7/44	P^{32}—36.2 mc. orally		18.4	6.5	64	19.4	9 wk.	16.5	6.0	50	2.25	22
3/21-23/46	Nitrogen mustard	30	18.0	5.9	57	17.0	4 wk.	13.1	4.7	45	2.9	7
10/16/46	P^{32}—12.5 mc. orally		19.5	6.4	63	24.0						
11/6/46	2.0 mc. I.V.		19.5	6.2	58	20.0	12 wk.	16.5	4.7	48	15.0	8
7/1/47	P^{32}—7.0 mc. I.V.		16.4	5.5	55		6 wk.	15.2	5.5	45	14.0	7
5. 53 F	No. 400496; Duration—2-3 yr.											
2/18/46	Phlebotomy 1,000 c.c. monthly											
	2/18-3/11 2,500 c.c.											0.5-0.7
	3/11-3/25 1,000 c.c.											
5/22/47	Nitrogen mustard	24	15.3	6.2	52	14.0	12 wk.	10.9	4.2	39		12

6. 65 M	No. 398601; Duration—2 yr.											
1/30/47	Phlebotomies 4,500 c.c.		22	6.3	68			17.9	5.5	53		0.5-0.7
3/1/47 }												
7/19/47 }	Fowler's solution		17.9	5.5	53		8 wk.	14.6	6.3	53		5
10/4/45	No treatment		16.3	6.7	62							
10/25/47	Phlebotomies—600 c.c. × 2		15.0	6.8	52							
10/29/47	Nitrogen mustard	32					3 wk.	12.2	5.6	42	3.5	8
7. 54 M	No. 413822; Duration—2 yr.											
8/1/47	Phlebotomies 2 × 500 c.c.		22.6	6.4	70	11.4	1 wk.	19.5	5.9	60		
8/24/47	Nitrogen mustard	28	19.5	5.9	60		12 wk.	16.2	5.0	54		0
9/5/47	Phlebotomy 2 × 500 c.c.											
10/24/47	Nitrogen mustard	28	18.5	5.3	59		6 wk.	14.8	4.5	51		3
3/16/48	P32—6.5 mc. I.V. and		14.5	4.8	50		8 wk.					0
	Phlebotomies 500 c.c. ea. wk. × 3							12.5	3.8	41		5
8. 55 F	No. 381989; Duration—10 yr.											
1939	Phlebotomies 1500 c.c. in 2 wk.											0.75
1939-1945	X-ray—2-3 yr.											
8/20/46	Nitrogen mustard preceded by phlebotomies 1,500 c.c.	24	14.6	7.3	54	13.0	4 wk.	10.7	4.8	39		7
9. 48 M	No. 270238; Duration—20 yr.											
	Phenylhydrazine, phlebotomies, (total body irradiation)											
7/43	Phlebotomies											
5/44	P32—18.6 mc. orally		12.0	5.7	46		4 wk.	13.1	5.4	47		1.5-2
12/5/44	P32—6.0 mc. orally		17.0	7.3	58							
1/2/45	6.0											6
3/8/45	5.0		16.8	6.4	56		1 mo.	15.4	6.1	56		6
1/22/46	Nitrogen mustard (phlebotomy)	24	15.2	6.2	50	7.1	4 wk.	13.2	5.3	45	.98	
							Apr. 1948	13.0	4.4	40	3.0	2.5 yr.‡
10. 55 F	No. 177125; Duration—9 yr.											
3/19/40	Phenylhydrazine 0.1 Gm. 2 × wk.		26	8.4	73	8.9		17.0	5.2	56	9.5	6 yr.
4/29/46	Nitrogen mustard phlebotomies 400 c.c. × 3	21.6	21.0	7.0	66	8.1	12 wk.	12.5	4.1	41	1.05	7 mo.
1/29/47	P32—(5 mc. I.V.)		19.0	7.2	62	5.2		13.4	4.1	39	2.8	16

Hct., hematocrit.

*Phlebotomies during two weeks following nitrogen mustard.

†Phlebotomies at monthly intervals.

‡Patient still in remission 10/25/49.

earlier level of 1.5 per cent in the following eight weeks. These changes are quite similar to those found in the earlier studies of lymphomas. The reticulocyte count may be considered a rather reliable index of the suppressive effect of the agent despite the pretreatment use of phlebotomy since it has been shown that within therapeutic levels venesection does not stimulate reticulocytosis in polycythemia vera.[10, 11] There was no evidence that therapy produced hemolysis in this group.

Two cases (Cases 3 and 7) are of interest because of the brief and clinically insignificant remissions produced by nitrogen mustard. Erythropoiesis was extremely rapid in these cases with reticulocyte counts ranging up to 6 per cent, but after treatment suppression below 2 per cent persisted only four weeks. In both cases the hematocrit reflected the suppressive influence of the nitrogen mustard over a period of two months, but in neither was it lowered below 51 per cent. It is noteworthy that in Case 3 the hematocrit was not reduced to 60 per cent prior to treatment as in other cases of the group. The fall in hematocrit was 15, 4, and 1 per cent following therapy as compared with an average fall of 9 per cent in the other cases of the group. In Case 7 the hematocrit was reduced to 60 per cent by venesection, but within twelve weeks phlebotomy was necessary because of active regeneration.

In this study we have had the opportunity to observe two patients with polycythemia vera of considerable duration who have subsequently shown evidence of a leucemoid blood picture. The implications of this development are sufficient to warrant summarizing the clinical course of one case since this patient also had a myelofibrosis demonstrated by bone marrow biopsy.

CASE 8.—This 55-year-old white Swedish housewife first sought medical aid elsewhere in December 1938 because of frontal headache, lassitude, and a sudden severe sharp right abdominal pain. Blood studies revealed the following: hemoglobin, 107 per cent; erythrocytes, 9,000,000; leucocytes, 20,000 per cubic millimeter. The differential count was: neutrophiles, 60 per cent; stabs, 13 per cent; metamyelocytes, 3 per cent; myelocytes, 1 per cent; lymphocytes, 19 per cent; and monocytes, 5 per cent.

In the following six weeks the patient had phlebotomies of 500 c.c. every week. X-ray therapy was directed to the spleen in February 1939 and at four- to six-month intervals until December 1945, when the patient was considered to have received her tolerance dose. At this time the patient had an episode of tarry stools. X-ray studies of the gastrointestinal tract did not reveal the cause of this hemorrhage. In the four months prior to her first visit to the University of Chicago Clinics, she had noticed progressive fatigue, exertional dyspnea, pain in the lower legs at rest, and dependent edema. Physical examination revealed a chronically ill, obese woman of ruddy complexion with congested mucous membranes. The chest was clear, no cardiac enlargement was present, blood pressure was 160/90, and the liver and spleen were not palpable. There were moderate varicosities and dependent edema of the lower extremities. Laboratory studies included an erythrocyte count of 7,310,000 per cubic millimeter, hemoglobin of 14.6 per cent, and hematocrit of 54 per cent. The leucocyte count was 13,250 per cubic millimeter, of which 75 per cent were segmented forms, 16 per cent lymphocytes, 1 per cent monocytes, 1 per cent basophiles, and 3 per cent myelocytes. The reticulocyte count was 1.3 per cent. Sternal marrow aspiration showed an active marrow with considerable myeloid activity.

The patient was prepared for nitrogen mustard therapy by three phlebotomies of 500 c.c. each. On Aug. 20, 1946, a course of four consecutive daily injections of 0.1 mg. per kilogram body weight (total of 24 mg.) was begun. In the following eight months the

erythrocyte count and hemoglobin levels remained within normal variations as shown on Table II. However, there were 8 per cent blast forms in the smear of April 3, 1947. In May, 1947, the patient developed weakness, nausea, vomiting, and diarrhea. When seen on June 4, 1947, she had scattered râles in the lung bases, the liver was enlarged and tender, and a moderately firm spleen extended to the umbilicus. Blood studies included: hemoglobin, 7.1 Gm. per cent; 3,200,000 erythrocytes per cubic millimeter; and 12,200 leucocytes per cubic millimeter. The differential count included 2 per cent blast forms, 6 per cent myelocytes, 14 per cent metamyelocytes, 65 per cent segmented neutrophiles, 11 per cent lymphocytes, 1 per cent monocytes, and 1 per cent basophiles with 6 erythroblasts per 100 leucocytes. Sternal marrow aspiration was unsuccessful twice. A surgical biopsy revealed a marrow consisting of a fibrogelatinous matrix with many sclerotic islands of reticular cells and megakaryocytes and occasional foci of myelocytes, erythroblasts, and plasma cells. Other diagnostic measures including gastrointestinal x-ray studies, urobilinogen excretion studies, liver and renal function tests, and stool examinations did not explain the sudden anemia. The anemia was progressive and numerous transfusions increased the blood count only moderately. The patient expired at home Aug. 8, 1947. This is the picture of myelofibrosis with a leucemoid reaction more closely resembling the agnogenic myeloid metaplasia of Jackson[12] than true myelogenous leucemia. An autopsy was not performed; hence, the possibility of a chronic myelogenous leucemia was not excluded.

The summary of the clinical course of Case 8 through the early years is quite typical of her disease. There had been active roentgen therapy and phlebotomies over a period of ten years. At the time we first examined the patient there was moderate evidence of immaturity in the peripheral blood picture. The remission induced by nitrogen mustard therapy was of eight months' duration. However, in the eighth month immature myeloid forms increased and in the tenth month a severe anemia became apparent. We cannot state what influence nitrogen mustard may have had on the development of this myelofibrosis, but we have seen a similar change in patients with polycythemia vera who were not treated with nitrogen mustard. The duration of the original disease and the interval of eight months from treatment to its appearance suggest that this development was not the result of nitrogen mustard therapy but rather a phase of the natural course of the disease.

The patient (Case 9) whose condition was of twenty years' duration has had the longest remission of the group. He showed a moderate hypochromic anemia following therapy with a delayed erythrocyte regeneration. Occasional immature myeloid forms appeared in the peripheral smear thirty months after therapy. This again is not interpreted as evidence of a change produced by nitrogen mustard, but this consideration must be evaluated in future observations.

DISCUSSION

This group of ten cases offers a limited opportunity to compare the efficacy of the various agents used in the control of polycythemia vera. Our limitation here arises not only from the small number of cases, but also from the characteristic variability of the activity of the disease. Falconer[13] reported an unusual spontaneous remission of eleven years. We have observed spontaneous remissions of two to five years in our cases. This factor has not often been recognized in reporting the use of certain therapeutic measures. In this group one patient (Case 9) is having a prolonged remission, greater than one is justified in ascrib-

TABLE II. SUMMARY OF PERIPHERAL BLOOD STUDIES IN CASE 8 (NITROGEN MUSTARD THERAPY)

DATE OF TREATMENT	HGB.	HCT.	R.B.C.	W.B.C.	NEUTRO-PHILE	METAMY-ELOCYTE	MYELO-CYTES	BLAST FORMS	LYMPHO-CYTES	EOSINO-PHILES	BASO-PHILES	MONO-CYTES	ERYTH-ROBLAST /100 W.B.C.	RE-TICULO-CYTES
8/13/46	14.6	54	7.3	13,000	75		3		16	4	1	1	0	1.3%
8/20-23/46		*Period of Nitrogen Mustard Therapy*							23	4	2	4	0	0.7% 5.0
9/10/46	10.2	39	4.62	6,050	67									
10/ 8/46	12.2		6.0	11,300	74	0	0	0	19	6	1	0	4	
12/12/46	13.8	47	6.2											
2/ 5/47	13.8	50	5.8											
4/ 3/47	13.8	44	5.8	14,000	62	0	0	8	19	2	6	3	2	
5/15/47	12.3		5.3	13,600	72	3	6	5	8	2	4	0	3/100	
6/ 4/47	7.1		3.2	12.200	65	14	6	2	11	0	1	1	5/100	3.0
6/13/47	5.7		2.1	15,100	69	14	6	2	4	2	2	0	14/100	
6/30/47	8.0		3.8	9,300	59	11	5	6	15	1	3		3/100	
7/11/47	10.0		3.2	11,000	49	16	3	3	22	1	2	4	3/100	0.8

ing to the therapeutic agent. Using uniform criteria of remission we have attempted to tabulate the response of this group of patients to various measures (Table III).

Phlebotomy has long been recommended in the control of thrombotic emergencies of polycythemia, but its usefulness as a therapeutic measure was first emphasized by Stephens and Kaltreider.[11] Their group included four cases of polycythemia vera. Following a series of bleedings to reduce the blood levels to normal, three patients had remissions of five, ten, and twenty-five months respectively; one required bleeding every four weeks. Similar experiences have been reported by others.[12, 13] Our group contains eight cases in which there is accurate information concerning the blood counts and frequency of phlebotomies (each of 500 c.c.). After the initial bleeding to bring the cell volume within normal limits, the period of remission was from two to eight weeks, or an average of 4.6 weeks. This means of control is considered relatively effective, but microcytosis and hypochromia may develop. Although the blood level is controlled by phlebotomy, distressing splenomegaly may develop.

Inorganic arsenic was introduced in the therapy of polycythemia vera by Forkner and co-workers.[16] They noted the response to Fowler's solution in six patients. Improvement was noted in ten to fifty days, and remissions were prolonged over many months by the use of a maintenance dose. Chronic use of the compound may be associated with neuropathy or pigmentary changes in the skin and later potentially malignant hyperkeratoses.

Since the report of Eppinger and Kloss[17] there have been many publications on the efficacy of phenylhydrazine hydrochloride including the recent statement by O'Connell and Johnson[18] that it is the most satisfactory agent. There are, in the literature, warnings of its acute hemolytic effect and irregular behavior in the aged and debilitated. Giffin and Conner[19] site two incidents of acute hemolysis as a cause of death in a group of forty-one cases. Stone and associates[20] and McNamara and Sansum[21] have commented on the toxic reactions. These reactions usually have occurred at the onset of therapy with administration of large doses. Results have been most satisfactory when doses of 0.1 or 0.3 Gm., two to three times weekly, have been used as a maintenance medication.

In this group of patients three received acetyl phenylhydrazine at intervals. One patient (Case 2) was given acetyl phenylhydrazine to initiate therapy on two occasions by her physicians; each resulted in acute hemolysis seven days following the initial dose and transfusions were necessary to support the patient. Two others (Cases 9 and 10) have been, at various times, on maintenance doses for periods up to six years with control of the peripheral blood levels in the normal range, but each has had progressive splenomegaly. It appears that acetyl phenylhydrazine is efficacious as a maintenance medication, except for failure to reduce the splenomegaly, but phlebotomy is the safest means of initially reducing the red cell mass.

The earlier efforts to utilize roentgen therapy in the control of polycythemia have been reviewed by Pack and Craver.[22] They record their observation of

TABLE III. A SURVEY OF THE RESPONSE OF TEN CASES OF POLYCYTHEMIA TO VARIOUS THERAPEUTIC MEASURES

CASE	PHLEBOTOMY*	FOWLER'S	ACETYL PHENYLHYDRAZINE	RADIOPHOSPHORUS			NITROGEN MUSTARD	
				DOSE	INTERVAL†	REMISSION (MO.)	INTERVAL†	REMISSION (MO.)
1	3 wk.	On med. 4 mo. Total remission 4 mo.					3 Phlebotomies 1st week	6 5
2	4 wk.		Two episodes acute hemolysis Transfusions				8 wk. 6 wk.	4 5
3				13 mc. P.O. 7 mc. I.V.		0 0	3 wk. 3 wk. 3 wk.	2 2 2.5
4				36 mc. P.O. 12 mc. P.O. 2 mc. I.V.	9 wk. 8 wk.	22 8	4 wk.	7
5	2-3 wk.						4 wk. normal 12 wk. lowest	12
6	2-3 wk.	On med. 2 mo. Total remission 5 mo.					3 wk.	8
7	2 wk.			6.5 mc. I.V.		0	0 6 wk.	0 3
8	3 wk.						4 wk.	7
9	6-8 wk.		Intermittent for 10 yr.	2 mc. 17 mc. 6.6 mc. 5 mc.	2 × wk. 4 wk. 4 wk. 4 wk.	6 6	4 wk.	30.0
10	8 wk.		6 yr.	5 mc. I.V.	16 wk.	16	4-12 wk.	7
Average	4.6 wk.				8 wk.	8.1 mo.	4.4 wk.	6.5 mo.

*Duration of response of phlebotomies of 500 c.c.
†Interval from date of treatment to lowest hematocrit.

remissions of six to twelve months following irradiation of long bones. Others report similar experiences.[23] Therapy was directed to the spleen only when palliative relief was required. A definite disadvantage of this method is that the dose required is of such intensity as to prohibit frequent repetition.[24]

The report by Sgalitzer[25] of satisfacory remissions in forty-two cases of polycythemia treated by total body irradiation and further remissions upon repeating therapy stimulated wide interest in this type of therapy. Reznikoff and Carty[26] have reported a group of twenty-two patients receiving forty-one treatments. Remissions of more than six months occurred following thirty-three of the forty-one treatments; six courses resulted in remissions with the exception that patients in the later stages of the disease generally do not respond well.

Since the first report by Lawrence[28] in 1940 of the use of radioactive phosphorus (P^{32}) as a source of internal irradiation for the control of polycythemia vera, there have been a series of confirmatory reports.[29-32] In these, satisfactory remissions of several months to three years or more resulted, with a latent period preceding the fall in erythrocytes of forty to ninety days. Erf[33] has commented that this latent period is to be expected because the life of the circulating erythrocyte is ninety to one hundred twenty days. This method will continue to be a matter of discussion for some years because of the chronicity of the disease. However, it may be well to note that while Reinhard and co-workers[32] speak of P^{32} as the treatment of choice, Hahn and Sheppard[34] emphasize the impracticality of irradiation therapy and allude to the protean nature of its complications.

Some reason for cautious consideration may be found in the recent report by Hall and Watkins[35] of the occurrence of seven cases of acute leucemia in polycythemia treated with P^{32}. Of the ten cases we are reporting, five patients received radioactive phosphorus in eight courses of treatment. The response was quite satisfactory in four patients. Two courses of 13 mc. orally and 7 mc. intravenously were ineffective in Case 3. The remaining six courses produced remissions which varied from six to twenty-two months, with an average remission for the group of 8.1 months. In keeping with previous studies, the interval of latency in response averaged eight weeks.

With this summary of the response to other forms of medication one may consider the comparative efficacy of nitrogen mustard therapy in the control of polycythemia vera. The most apparent advantage is the prompt suppression of erythropoiesis. This is demonstrated by a decrease in reticulocytosis and a progressive decline in the hemoglobin, erythrocytes, and hematocrit which reached the lowest level at about 4.4 weeks after treatment. It has been mentioned that the average remission to single phlebotomies was 4.6 weeks after a series of venesections had brought the hematocrit within normal limits. Hence, with the program of pretreatment phlebotomies the disease is controlled from the time of treatment without a latent period in which the hematocrit may rise. The decreased threat of thrombosis accompanying this prompt response is a distinct clinical advantage.

A clinical response of 6.5 months is sufficiently long to be physiologically and economically sound to the patient. Nitrogen mustard failed to produce a remission of three months or longer in two patients. In one of these (Case 3) two courses of radioactive phosphorus were ineffective. However, both patients had very active disease as evidenced by the reticulocytosis. This may be a very significant corollary to the observation that in animals with the hemopoietic system in a regenerative phase, as produced by phenylhydrazine administration or repeated phlebotomy, there is a remarkable increase in the resistance of the marrow to toxic agents such as radiation or nitrogen mustard.[36] If this mechanism is active in the human being it will explain the brief remission observed in two of our patients (Cases 3 and 7). We have considered the feasibility of more intense therapy in cases of this type, but it is unlikely that the myelogenous and clotting elements will show this resistance phenomenon.

In all patients who had a hematologic remission, a prompt alleviation of clinical symptoms followed. This clinical and hematologic remission may be continuous if one uses regeneration to a hematocrit of 55 per cent as the criterion of relapse (Case 1) and institutes therapy promptly.

Consideration must be given to certain disadvantages and potential complications. The immediate toxic reaction of nausea and vomiting following the injection of nitrogen mustard was considerably less in this group than in our experience with lymphomas. This reaction was slight in seven subjects, lasting but two to four hours with little vomiting. The possibility of aggravating the inherent tendency of polycythemia vera toward leucemia,[35-39] or toward myelofibrosis with a leucemoid reaction,[12, 40-42] by treatment with nitrogen mustard must be considered. Two cases were cited (Cases 8 and 9) in which there developed evidence of a leucemoid reaction. It is to be noticed that these patients, with conditions of ten and twenty years' duration respectively, are in a stage of the disease in which this change may occur spontaneously.[41-44]

Wilkinson and Fletcher,[4] and recently Woodruff,[5] have reported unsatisfactory response in three of four cases of polycythemia vera. It must be recognized that they used single small doses of nitrogen mustard at intervals of several days. This method proved unsatisfactory in our hands in the treatment of lymphomas and leucemias.

SUMMARY

A group of ten cases of polycythemia vera is presented with a brief review of earlier therapy as a basis for evaluating the response to nitrogen mustard therapy. The standard course of 0.1 mg. methyl-*bis*(β-chloroethyl) amine hydrochloride per kilogram body weight intravenously on four consecutive days, given in patients prepared by phlebotomy, reduced the average hematocrit from 59 per cent to 44 per cent in 4.4 weeks. A hematocrit of 55 per cent was used as the criterion for the onset of relapse. Two patients relapsed within four months, two within four to six months; four patients had remissions of six to eight months, and two had remissions of eight months or longer. The average period of remission was 6.5 months. Two patients with cases of ten and twenty

years' duration developed leucemoid reactions after remissions of seven and thirty months.

The prompt hematologic and clinical response is considered advantageous and warrants extended observation of this method of therapy.

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PROGESTERONE AND ANHYDROHYDROXYPROGESTERONE: A COMPARATIVE STUDY OF ORAL ADMINISTRATION

WILLIAM BICKERS, M.D.
RICHMOND, VA.

AN EFFECTIVE, orally administered, progestational hormone has long been sought. It is widely held that progesterone administered orally is rendered biologically inert. An extensive survey of the literature does not reveal any well-controlled study in human beings to support this widely held concept. Contrarily, it has been recently shown by Dorfman[1] that progesterone given orally is followed by the excretion of significant amounts of pregnanediol in the urine. While this is not conclusive proof that oral progesterone is biologically active, it does suggest that progesterone may be metabolized in the human being following oral administration in a manner comparable to that following the intramuscular route. In a single case report by Allen[2] it was shown that oral progesterone in a dose of 200 mg. led to an increased excretion of pregnanediol in the urine.

In 1944 Corner[3] reported his observations upon the absorption and utilization of progesterone in the rhesus monkey. He utilized the well-known delaying effect of progesterone upon the onset of estrogen withdrawal bleeding as the testing method. From the experiment it was concluded that the effective sublingual dose of progesterone was approximately four to five times that of the intramuscular dose. In a recent report by me[4] it was shown that whereas 10 mg. of progesterone given intramuscularly daily for five days approximates the minimal effective dose for the consistent production of a well-developed progestational endometrium in the human being, a much larger dose is necessary when given sublingually. The minimal effective dose of sublingual progesterone in the human being as determined by endometrial response is approximately 90 mg. per day in divided dosage over a period of five days. It must be emphasized, however, that the progestational response of the endometrium is obtained only following adequate preparation of the endometrium with estrogen. Preliminary priming of the endometrium with estrogen over a period of ten to fifteen days is necessary for the consistent and efficient utilization of sublingual progesterone.

Anhydrohydroxyprogesterone (pregneninolone) has a progestational effect upon the endometrium after oral administration.[5] Salmon[6] has stated that 105 to 540 mg. of anhydrohydroxyprogesterone orally administered over a period of three to eight days is required. Neustaedter[7] reports that 900 mg. over a period of twelve days induces only a mid-luteal phase in the endometrium. Allen and Heckel,[8] and Zondek and Rozin,[9] also, have indicated that 80 mg. over a period of five to six days may be the minimal effective dose.

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MATERIALS AND METHODS

Dose Equivalents of Progesterone and Anhydrohydroxyprogesterone.—

Test Subjects: There were two groups of patients used for this comparative study: four patients under the age of 35 years recently subjected to bilateral oophorectomy and nine patients under 35 years of age with secondary amenorrhea. Each of the patients in these study groups had been tested previously for endometrial response by the oral administration of estrogen over a period of twenty days, supplemented by progesterone either administered intramuscularly or sublingually during the last five days of estrogen administration. Estrogen withdrawal bleeding had been induced previously in all test subjects by the administration of 40,000 I.U. (International units) of mixed natural estrogen (Urestrin*) daily for twenty days. This dose will consistently induce estrogen withdrawal bleeding. All had been found to have a responsive endometrium, albeit there was the expected individual variation in degree of response in different patients.

RESULTS

Dose Equivalents of Oral Progesterone and Oral Anhydrokydroxyprogesterone.—There were twelve cycles in which the effect of orally administered progesterone were studied. Three bleeding cycles were studied in patients recently castrated by surgery and nine cycles in patients with secondary amenorrhea of eight to twenty-four weeks' duration. Since the minimal sublingual dose of progesterone was previously determined to be approximately 90 mg. daily for five days following estrogen priming, it was decided to start these patients on swallowed (oral) progesterone at twice that dose. Accordingly the first castrated patient was given 180 mg. of progesterone orally in divided doses daily for five days following a preliminary priming with estrogen in the form of oral mixed natural estrogen, 4,000 I.U. daily for twenty days. This dose has been shown to be insufficient to consistently induce withdrawal bleeding. The same dose schedule was used in four induced cycles on the patients with secondary amenorrhea.

Castrated Patients—Effect of Oral Progesterone: One of the castrated patients was treated with 180 mg. of progesterone orally in divided doses daily for five days. Withdrawal bleeding occurred within eighty hours after completing treatment. In two cycles 120 mg. of progesterone were administered daily for five days and was followed by a well developed progestational endometrium in both cases. The patient who received 180 mg. had an endometrial response little different from those who had been subjected to the lesser doses. Edema of the stroma, basal migration of the nuclei in the glandular epithelial cells, and good secretion in the lumen of the glands were seen. (Fig. 1.)

Patients With Secondary Amenorrhea—Effect of Oral Progesterone: In nine cycles induced by oral administration of mixed natural estrogen (4,000 I.U. daily for twenty days) plus oral progesterone (120 mg. daily for five days) withdrawal bleeding occurred within seventy-one hours after treatment in all patients. Specimens of endometrium obtained by the suction curette

*Urestrin (The Upjohn Company, Kalamazoo, Mich.) was the mixed natural estrogen used in this study. The progesterone was supplied by The Upjohn Company and the anhydrohydroxyprogesterone was the commercial product known as Pranone (Schering Corporation, Bloomfield, N. J.).

Fig. 1.—The cytoplasm of the epithelial cells is filled with secretion. The nuclei are for the most part basal. The stroma is highly edematous and there are foci of decidua-like reaction. The reaction is that of a fully mature progestational phase. (×450)

on the first day of the withdrawal bleeding showed a proliferative phase endometrium in two, a transitional type of endometrium in one, and a moderate to good progestational response in six. A full progestational endometrium (plus 4) was observed in five of the tissues.

Anhydrohydroxyprogesterone.—Two of the castrated patients and six of the patients with secondary amenorrhea used in the oral progesterone study cooperated in a second cycle of treatment using anhydrohydroxyprogesterone in the place of progesterone for oral administration. The eight patients were given a preliminary dose of mixed natural estrogen, 4,000 I.U. daily for twenty days, a dose insufficient to induce withdrawal bleeding consistently. Anhydrohydroxyprogesterone in a dose of 120 mg. in divided dose daily was given for five days during the last five days of the estrogen administration.

Castrated Patients—Effect of Oral Anhydrohydroxyprogesterone: Endometrial biopsy was taken by suction curette within twenty-four hours of the onset of withdrawal bleeding in the eight cycles under study. Withdrawal bleeding occurred within six to eighty-four hours of treatment in all patients. The endometrium from one castrated patient, Fig. 2, showed a progestational response which might be classified as plus 3. It will be noted that although there is good invagination of the glandular epithelium, there is less edema of the stroma, no tendency to decidua-like formation in the interstitial cells, and a minimal amount of secretion in the lumen of the glands. This histologic picture was rather typical of the specimens of endometrium obtained in the anhydrohydroxyprogesterone-treated group. The endometrium obtained from the other castrated patient showed a similar pattern.

Patients With Secondary Amenorrhea—Effect of Oral Anhydrohydroxyprogesterone: In the secondary amenorrhea group there were two specimens of endometrium which failed to show any progestational response following the foregoing treatment schedule. The three specimens which did show a progestational effect had good epithelial invagination in all sections studied, but the secretion in the lumen of the gland was minimal. There was a minimal amount of edema in the stroma and no decidua-like formation.

CONCLUSIONS

1. Progesterone administered orally to the human being is biologically active in a dose of 120 mg. daily over a period of five days when administered after preliminary priming of the endometrium with mixed natural estrogen in a dose of 4,000 I.U. daily for twenty days. A progestational response of varying degree can be induced in patients who have had a bilateral oophorectomy or who have amenorrhea secondary to functional ovarian failure. The endometrial response may be that of a fully developed progestational endometrium. In most cases there is a marked invagination of the glandular epithelium, basal migration of the epithelial cell nuclei, abundant secretion in the lumen of the glands, edema of the stroma, and a tendency toward decidua-like change in scattered areas throughout the stroma.

Fig. 2.—The cytoplasm is poorly filled with secretion. There is no secretion in the lumen and no evidence of decidua-like reaction. The nuclear migration toward the basal portion of the epithelial cells is well demonstrated, however, and the infolding of the glandular epithelium is typical of a mid-luteal phase. (×450)

2. Anhydrohydroxyprogesterone is an effective oral progestational hormone in a dose of 120 mg. daily when administered during the last five days of a twenty-day period of estrogen priming. Although a progestational endometrium is induced, the secretory response in the endometrium appears to be less than that following the oral administration of progesterone. Invagination of the glandular epithelium and migration of the nuclei is comparable with that seen after progesterone, but secretion in the gland lumen, edema in the stroma, and tendency toward decidua-like cell formation in the stroma is absent.

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ACUTE EFFECT OF 4-AMINOPTEROYLGLUTAMIC ACID ON BLOOD LYMPHOCYTES AND THE LYMPHATIC TISSUE OF INTACT AND ADRENALECTOMIZED MICE

JEAN H. DOUGHERTY, M.D., AND THOMAS F. DOUGHERTY, PH.D.
SALT LAKE CITY, UTAH

ADMINISTRATION of folic acid antagonists results in a depression of hematopoiesis in all animal species studied. The most pronounced action of these compounds is on the bone marrow, although several reports indicate that there is also a very definite effect on the lymphatic system. A decreased number of circulating lymphocytes follows chronic administration of various of the antagonists or the feeding of a folic acid-free diet in mice[1] and in guinea pigs.[2] Lymphopenia was observed as early as the twelfth hour after a single large dose of 4-aminopteroylglutamic acid (Aminopterin) in rats[3] and after forty-eight hours in mice.[4] Atrophy and degenerative changes in the various lymphatic structures were found by several observers following both short- and long-term administration of the various antagonists. A decrease in the size of lymph nodes, spleens, and thymi of rats was observed by Phillips and Thersch[3] following a single massive dose of Aminopterin. After a five-day period of injection of this compound, Ritter and Oleson[5] found acute degenerative changes in the spleens and thymi of rats and an increased amount of connective tissue in these organs. Animals bearing local tumors and treated with varying amounts of Aminopterin had extremely atrophic spleens.[6] The decrease in size of these organs was due to a reduction in the amount of lymphatic tissue. Another group of investigators[1] found no consistent change in the amount of lymphatic tissue of the spleens of mice which had been on a folic acid-free diet supplemented with a crude antagonist.

It has been demonstrated that the level of circulating lymphocytes and the size of lymphatic organs may be influenced by pituitary and adrenal cortical secretions.[7, 8, 9] Furthermore, it is now clear that nonspecific stimuli of various types may increase adrenal cortical secretions which in turn results in a decrease in the number of blood lymphocytes and an acute involution of lymphatic organs.[9] The following experiments were designed to ascertain whether Aminopterin produced a diminution in blood lymphocytes and a reduction in the mass of lymphatic tissue directly or through pituitary-adrenal cortical mediation.

METHODS

Seventy-five CBA mice 16 weeks of age and weighing approximately 20 grams were used in this study. The animals were fed ad libitum on dog chow (Purina) supplemented with calf meal (Larro) before and during the course of the experiment and were maintained in constant temperature quarters.

From the Departments of Pathology and Anatomy, and the Division of Oncology, University of Utah School of Medicine.

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Aminopterin was administered intraperitoneally in saline solution daily for a period of five days to both intact and adrenalectomized mice. The latter animals were adrenalectomized one day prior to the first injection of Aminopterin and were maintained on daily subcutaneous injections of 0.025 ml. of an oily solution of desoxycorticosterone acetate. Both groups of animals received 0.2 ml. daily of a solution which contained 0.02 mg. of Aminopterin (1.0 to 1.2 mg. per kilogram). This amount of compound was lethal to 16-week-old animals; death occurred after seven to ten days of treatment. This large dose was selected because it resulted in a lymphopenia and lymphatic tissue atrophy within a period of time during which adrenalectomized mice survive and remain in good condition.

The animals were weighed daily. Tail vein blood was used for all blood counts. Total white cell and differential counts were determined on twelve of the intact, treated mice at three, six, and twenty-four hours after the initial injection of Aminopterin. Similar counts were performed on the blood of both the intact and adrenalectomized animals twenty-four hours following the administration of the first, third, and fifth doses of the compound.

The animals were sacrificed on the sixth day (twenty-four hours following the final injection) by severing the spinal cord. At autopsy, the weights of the inguinal, axillary, and mesenteric lymph nodes, thymi, spleens, and adrenal glands were determined. These tissues were either fixed in Zenker's solution for histologic study or prepared for nitrogen analyses. The fixed tissues were stained with either hematoxylin and eosin or methyl green pyronin. One of each pair of adrenal glands was fixed in Formalin and stained with sudan IV. Nitrogen analyses were determined by the micro-Kjeldahl method. Air-dried bone marrow imprints from the sternum were also obtained from all animals.

Two groups of control animals were used in these experiments. The first group consisted of 16-week-old intact animals. A second group was made up of 16-week-old nontreated adrenalectomized mice which were sacrificed six days following operation. This group of adrenalectomized mice was thus allowed to live for the same period of time following operation as the adrenalectomized treated mice.

RESULTS

Both groups of Aminopterin-treated animals lost considerable weight. The weight loss began within forty-eight hours after the initial injection at which time the mice began to show the rough coat and diarrhea which are characteristic of animals treated with folic acid antagonists or kept on a folic acid-free diet. At autopsy, the mean weight loss was 3.5 grams and 2.5 grams for the intact and adrenalectomized animals respectively (Table I). The difference in weight loss between the two groups was not significant. It may be assumed that the weight loss of the Aminopterin-treated adrenalectomized animals was due to the treatment since untreated adrenalectomized controls did not show any weight loss during the same period after adrenalectomy.

Blood Findings.—The effect of a single dose of Aminopterin on the white blood cells is illustrated in Fig. 1. The number of circulating lymphocytes began to fall within three hours and reached its lowest level six hours after the injection of Aminopterin. Within twenty-four hours after injection, the lymphocyte count was again within the normal range.

In contrast to the results obtained following a single injection of Aminopterin, continued daily administration of this compound to intact mice resulted in a progressive decline in the level of blood lymphocytes (Fig. 2). The Aminopterin-treated adrenalectomized animals, on the other hand, did not develop a statistically significant lymphopenia at any time (Fig. 2). A sustained and statistically significant lymphocytosis was found in the nontreated adrenalecto-

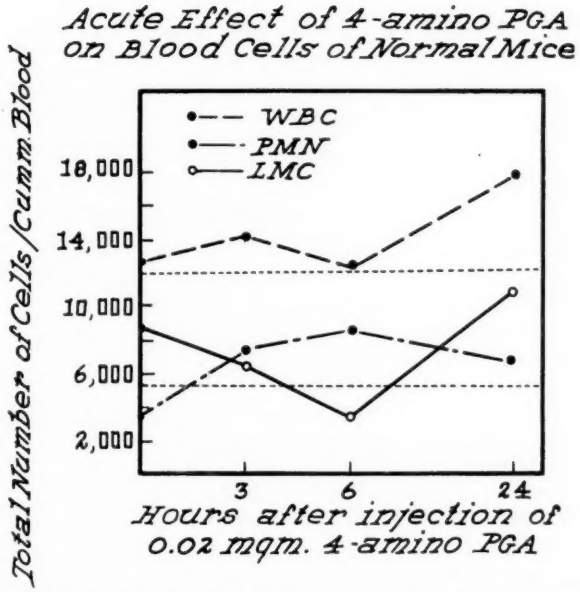

Fig. 1.—Alterations in numbers of leucocytes of intact mice following a single injection of 4-aminopteroylglutamic acid. The values plotted at zero time are control values determined on the blood samples of 150 mice of the CBA strain. The dotted lines are placed at one standard deviation on each side of the mean of the lymphocyte value of control intact mice.

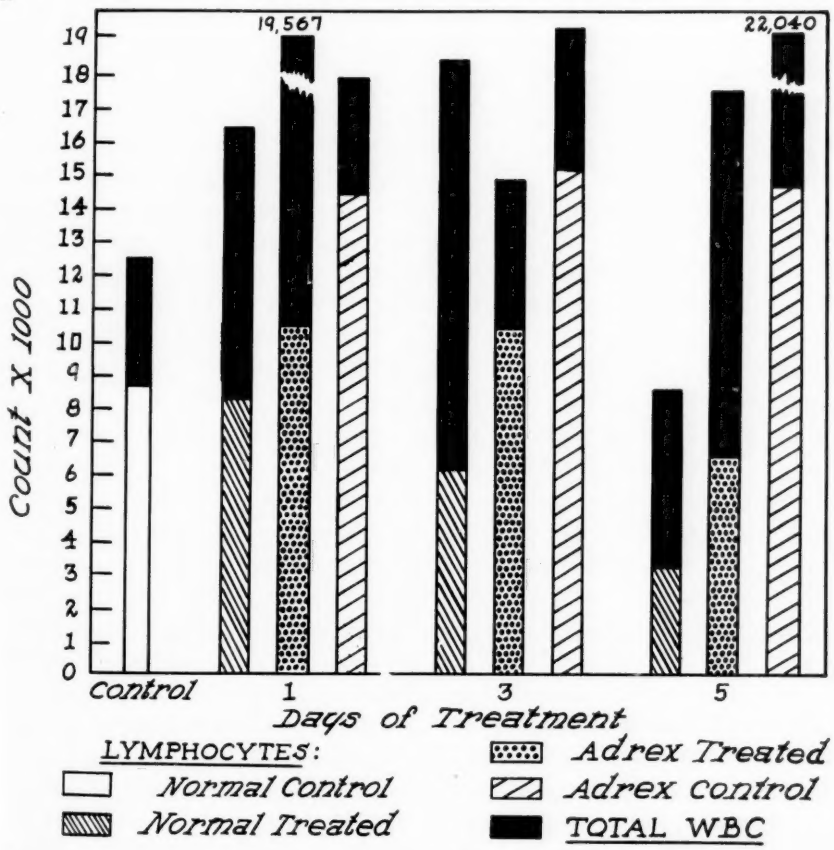

Fig. 2.—Comparison of the numbers of leucocytes of intact and adrenalectomized mice given Aminopterin with the leucocyte values of intact and adrenalectomized nontreated animals.

TABLE I. EFFECT OF DAILY INJECTION OF 0.02 MG. OF 4-AMINO PGA ON [illegible]GHT AN

GROUP	TREATMENT	NUMBER OF MICE	WT. LOSS (GM.)	LYMPHATIC TISSUE N_2 (MG.)	INGUINAL NODE		MESEN
					WT. (MG.)	% CHA	T. (MG.
I	Intact, control	19	--------	20.5 ± 3.4‡	31.5 ± 7.7	--	4.9 ± 2
II	Intact, 4-amino PGA	22	3.5 ± 1.4	12.3 ± 3.5	19.4 ± 6.9	-3	2.5 ± 3
III	Adren., control	10	0.4 ± 0.2	28.5 ± 3.1	40.5 ± 4.6	+3	3.0 ± 1
IV	Adren., 4-amino PGA	24	2.5 ± 0.9	21.8 ± 4.1	37.7 ± 11.2	+2	7.4 ± 4

*The mean organ weights and nitrogen values are expressed per 100 grams fresh tissue of initial body weight.

†Per cent weight change is from Group I, control.

‡Standard deviation from the mean.

mized animals (Fig. 2). The changes in the polymorphonuclear leucocytes which follow Aminopterin treatment are outside the scope of the present report.

Organ Weights.—The mean organ weights of the four groups of animals and the mean percentage weight differences of the organs of each of the experimental groups as compared with the intact control mice are summarized in Table I. The absolute weights are not given since they so closely paralleled the relative weights. There was no significant difference in the response of the organs of either sex to Aminopterin treatment.

The administration of Aminopterin to the intact animals (Group II) resulted in significant loss in weight of all lymphatic organs studied with the exception of the mesenteric node. This structure did not deviate significantly from the control value. The thymi lost by far the greatest amount of weight. The adrenal glands of the intact treated mice underwent a statistically significant increase in weight (45 per cent) during the period of treatment.

The lymphatic organs of the adrenalectomized treated animals (Group IV) did not undergo weight loss with the single exception of the thymi. These organs lost only about a third as much weight as did the thymus glands of the intact treated mice. The lymphatic organs of the adrenalectomized control mice (Group III) underwent significant hypertrophy ranging from a 20 per cent to a 50 per cent weight gain over values of the intact control animals. The adrenalectomized treated mice (Group IV) also had similarly hypertrophic lymphatic organs with the exception of the thymi which, as mentioned, actually lost weight following treatment.

Nitrogen Content of Lymphatic Organs.—The data obtained from the analysis of the nitrogen contents of the total samples of lymphatic tissue demonstrated that the weight changes were actually due to alterations in protein content. The differences in nitrogen content (Table I) of the lymphatic tissue of the various groups of animals correlated closely with the weight changes of the individual lymphatic organs. The nitrogen contents of the tissues of treated intact animals were significantly less and those of the adrenalectomized controls were significantly greater than those of the nontreated control group (Table I). Aminopterin treatment of the adrenalectomized animals did not result in a loss of lymphatic tissue nitrogen as compared with the intact control group. However, unlike the nontreated adrenalectomized controls, the lymphatic tissue nitrogen of the Aminopterin-treated adrenalectomized animals did not increase.

A ON E[illegible]IGHT AND ORGAN WEIGHTS* OF INTACT AND ADRENALECTOMIZED MICE

NODE	MESENTERIC NODE		THYMUS		SPLEEN		ADRENAL	
% [illegible] CHA[illegible]	T. (MG.)	% WT. CHANGE	WT. (MG.)	% WT. CHANGE	WT. (MG.)	% WT. CHANGE	WT. (MG.)	% WT. CHANGE
--	[illegible]4.9 ± 23.3	---	178.0 ± 26.5	---	277.0 ± 48.6	---	16.5 ± 1.8	---
-[illegible]	[illegible]2.5 ± 30.2	+ 7	58.3 ± 37.2	-67	193.5 ± 43.3	-30	23.7 ± 4.7	+45
-[illegible]	[illegible]3.0 ± 19.0	+27	213.0 ± 42.2	+20	414.0 ± 54.9	+50	----------	---
-[illegible]	[illegible]7.4 ± 40.9	+50	139.6 ± 36.9	-21	360.0 ± 87.7	+30	----------	---

Organ Histology.—The most prominent alterations in the lymphatic tissue of the intact Aminopterin-treated animals consisted of a loss of lymphocytes from the various lymphatic organs and evidences of increased lymphocytolysis. The term "lymphocytolysis," as used here, denotes increased pycnosis and degenerative changes in small and medium-sized lymphocytes. There were no cytologic alterations in the immature lymphocytes. Fewer mitotic figures were observed in the lymphatic organs of treated animals as compared with the number found in the organs of intact control mice. Mitotic counts on the thymic cortices of four of the treated animals showed an average of 1.5 mitotic figures per 1,000 cells as compared with the normal mean of 15.6.

The cortices of the lymph nodes and white pulp of the spleens contained greatly decreased numbers of secondary nodules. Although there did not appear to be any decrease in the number of immature lymphocytes in these structures, it is likely that the total number was diminished since these cells are found mainly in the secondary nodules. The mesenteric lymph nodes showed widened interstitial spaces containing a pale-staining fluid and swollen reticuloendothelial cells. This increased amount of fluid might explain the absence of weight loss in these nodes, in spite of the narrowed cortices and increased cellular destruction.

The lymphatic tissue of the adrenalectomized treated mice did not show the alterations already described. Instead, the microscopic appearance of the organs of these animals did not differ essentially from that of the organs in the adrenalectomized controls. The lymphatic tissue was densely packed with lymphocytes and there were numerous mitotic figures. The lymph nodes and white pulp of the spleens contained many secondary nodules filled with immature and developing lymphocytes.

The relative size and comparative amounts of cortex and medulla of the thymi of the four groups of mice are illustrated in Fig. 3. It may be seen that the cortex of the thymus of an intact Aminopterin-treated animal (Fig. 3, *B*) is almost absent as compared with the amount of cortical substance present in an intact control (Fig. 3, *A*). The thymus of a nontreated adrenalectomized animal (Fig. 3, *C*) has a hypertrophied cortex as compared with that of the intact control mouse. The amount of cortical substance of an Aminopterin-treated adrenalectomized animal (Fig. 3, *D*) is less than that found in either untreated intact or adrenalectomized mice but is not as atrophic as that found in intact Aminopterin-treated animals.

The red pulp of the spleens of mice is normally a site of erythropoiesis and megakaryocyte production. In both the Aminopterin-treated intact and adrenalectomized animals there were extremely few megakaryocytes and an almost complete absence of developing red cells. Further, there was a hyperplasia of the reticuloendothelial cells of the red pulp of the adrenalectomized treated animals.

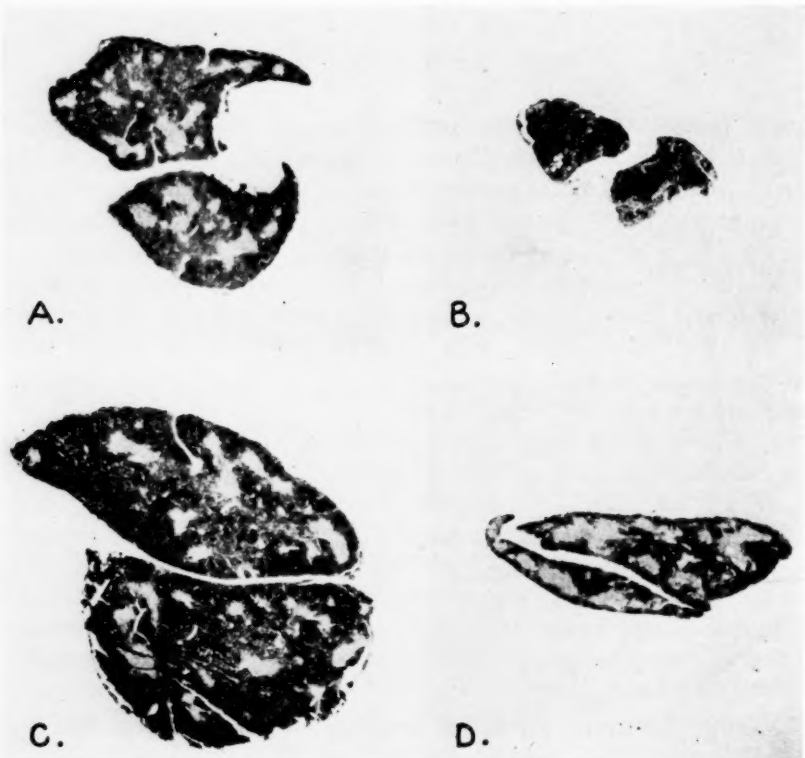

Fig. 3.—The effect of Aminopterin on the thymi of unoperated and adrenalectomized mice (×12). *A*, Untreated intact control. *B*, Intact Aminopterin-treated animal following five injections at daily intervals. The cortical portion is almost abolished. *C*, Nontreated adrenalectomized control animal five days following operation. Extensive increase in cortex as compared with the intact nontreated control. *D*, Aminopterin-treated adrenalectomized mouse following five injections at daily intervals. Cortex is smaller than that of either intact or adrenalectomized control animal but larger than that of intact treated animal.

The zona fasciculata of the adrenal glands of treated animals was thickened and the sinusoids were dilated. The adrenal cortical cells of the fasciculata were large and had a vesicular cytoplasm. Within three hours following injection of the initial dose of Aminopterin there was a complete depletion of the sudanophilic material of the zona fasciculata.

The bone marrow alterations will be described in more detail in a subsequent report. Identical changes were observed in the bone marrows of both groups of Aminopterin-treated animals. There was a decreased number of red cells of all stages of maturity and a "shift to the left" of the granulocytes with increased vacuolation and nuclear segmentation in the immature granulocytes

and stem cells. This premature nuclear segmentation was reflected in the mature polymorphonuclears which contained more nuclear lobes than were found in the cells of untreated animals.

DISCUSSION

Evaluation of the results of investigations concerning the influence of various agents on the growth of lymphatic tissue must take into account adrenal cortical control of this tissue. The results of recent studies indicate that certain adrenal cortical steroids have the capacity to bring about a dissolution of lymphocytes.[10] Further, the ability of numerous nonspecific substances to produce accidental involution of lymphatic tissue is known to be due to the capacity of these agents to stimulate adrenal cortical secretion.[8] Although most agents which bring about accidental involution apparently do so by hormonal means, there are others such as x-rays and nitrogen mustards which, in addition, have a direct lymphocytolytic effect.[8, 11]

The data presented in this report demonstrate that Aminopterin, like other toxic substances, stimulates adrenal cortical secretion. A single injection of Aminopterin in the dose employed produced a lymphopenia of brief duration,[8] depletion of adrenal cortical sudanophilic material, and dissolution of lymphocytes in the tissues. These three criteria have been shown previously to be an indication of a sudden increase of adrenal cortical secretion.[8]

The fact that Aminopterin has the capacity to act as a nonspecific stressful agent should not be taken to indicate that the sole effect of this compound (or any other agent) on lymphatic tissue is mediated by indirect hormonal mechanisms. The distinction between direct and indirect effects of Aminopterin has been made by administering this compound for a period of several days to intact and adrenalectomized animals. Although the circulating lymphocytes and nitrogen contents of lymphatic organs did not diminish in the adrenalectomized treated mice when compared with intact control values, they did not reach the increased level found among the adrenalectomized control animals. Thus, it is clear that whereas Aminopterin did not produce accidental involution in the absence of the adrenal cortex, it did inhibit the rate of growth of lymphatic tissue which follows adrenalectomy. An analysis of the data indicate that this decrease of over-all growth of lymphatic organs in adrenalectomized treated animals was primarily due to an inhibition of the growth rate of the thymus. The increment in weight of the lymph nodes of adrenalectomized treated animals was not diminished. The fact that the number of blood lymphocytes did not attain the level found in adrenalectomized animals may be related to the inhibition of thymus growth.

At present the only known action of adrenal cortical secretion on lymphatic tissue growth is its capacity to bring about accidental involution by lymphocytolysis.[10] It is not strictly correct, therefore, to assume that because a particular agent does not produce acute or accidental involution in the absence of the adrenal cortex it does not play a role in influencing lymphatic tissue growth. The roles played by nutritional constituents in lymphatic organ growth are particularly difficult to evaluate since dietary restriction alone produces acute in-

volution through pituitary adrenal cortical secretions.[12] The methods used in the present investigation may be of value in approaching other similar problems related to lymphatic organ growth.

The differences in the reaction of the various lymphatic organs to Aminopterin administration cannot as yet be explained. However, it is important to emphasize that general conclusions concerning lymphatic tissue should not be drawn from a study of only one lymphatic structure. Other data obtained in this laboratory indicate that the thymus is far more labile in its reactions than are other lymphatic organs.

The more direct action of Aminopterin on the inhibition of the growth of the thymus in adrenalectomized animals may be related to its capacity to inhibit folic acid. The mechanism of action of folic acid on the metabolism of blood cells is not known. It has been suggested that folic acid inhibitors depress the growth of myeloid and erythroid cells by bringing about a maturation arrest at an early stage of development of these cells.[1, 2] The changes observed in the present studies confirm the alterations described in the bone marrow of mice by other investigators.[1] It is apparent from the evidence presented in this report that pituitary and adrenal cortical secretions are not involved in the action of Aminopterin on bone marrow cells.

The cytologic changes in lymphatic tissue seen after Aminopterin administration to intact mice were similar to those produced by adrenocorticotropic hormone or adrenal cortical hormones.[10] However, the direct effect of Aminopterin on the thymi of adrenalectomized mice did not result in cytologic changes which yielded information concerning the mechanism of action of folic acid on lymphocytopoiesis.

The progressive growth of some acute leucemias in human beings and lymphomata in various experimental animals is inhibited by administration of Aminopterin in near toxic dosages.[4, 6, 13-15] Although this compound in large amounts probably acts as a stress agent, it cannot be inferred from the data presented in these studies that its favorable action in these conditions is by a hormonally mediated destructive action on the malignant cells. If this were so, all stressful agents would be expected to have a similar beneficial action on leucemia. Further, Aminopterin would also have an effect on the cells of chronic lymphatic leucemia. Examination of the immature lymphocytes in the organs of normal Aminopterin-treated mice revealed no alteration that would explain the sensitivity of immature malignant lymphocytes to this compound.

SUMMARY

The lymphopenia and acute atrophy of lymphatic tissue which were observed following administration of a lethal dose of 4-aminopteroylglutamic acid to mice did not occur when the animals were adrenalectomized prior to treatment. Therefore, it is suggested that these changes in lymphatic structures are probably hormonally mediated because of the nonspecific toxic action of the compound and the resulting stimulation of the pituitary and adrenal cortical secretions. This is substantiated by the observation that adrenal cortical hyper-

trophy and depletion of adrenal sudanophilic material followed the injection of a large dose of Aminopterin. However, the lymphocytosis and hypertrophy of the thymus characteristically observed after adrenalectomy were not found in the adrenalectomized Aminopterin-treated animals. It is probable, therefore, that this compound has both an indirect hormonally mediated as well as a direct inhibitory action on the lymphatic system.

In contrast to this dual action of Aminopterin on the lymphatic system, the effect of this drug on myelopoiesis was not mediated wholly or in part by pituitary and adrenal cortical secretion.

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ARTIFACTUAL VALUES OF SERUM PRECIPITABLE IODINE

EVELYN B. MAN, PH.D., AND JOHN P. PETERS, M.D.
NEW HAVEN, CONN.

IF THE proteins of serum are precipitated with zinc hydroxide and the precipitate is washed, inorganic iodine which has gained access to the blood from the use of Lugol's solution, potassium iodide, iodized salt, or Furmethide is removed from the precipitate, leaving only the protein-bound iodine, which is composed almost entirely of thyroid hormone.[1] This *serum precipitable iodine* (SPI) is, therefore, under ordinary circumstances, a most reliable criterion of the activity of the thyroid gland. Certain organic compounds of iodine which are used for diagnostic or therapeutic purposes, however, are precipitated with serum proteins and are not removed by washing. Spuriously high values for SPI are, therefore, found in patients who have received such compounds. This paper deals with an attempt to ascertain the duration of the effects on SPI of the administration of Priodax [β-(4-hydroxy-3,5-diiodo-phenyl)-α-phenylpropionic acid], Diodrast (3,5-diiodo-4-pyridone-N-acetic acid diethanolamine), and Lipiodol, three compounds commonly employed for diagnostic purposes. It is probable that other similar compounds and diiodoquinone have similar effects.

In a study of SPI, now in preparation for publication, it has been established that the statistical limits of the normal range are 3.8 to 7.8 gamma per cent. Occasional apparently euthyroid individuals have SPI's as low as 3.6 and as high as 7.8 gamma per cent.[2]

MATERIALS AND METHODS

Priodax.—The SPI of seven patients was measured before and at intervals after the oral administration of 3 grains of Priodax for roentgen visualization of the gall bladder. None of the patients had any demonstrable disorder of function of the thyroid gland, kidneys, liver, or gall bladder. The results of these studies are presented in Fig. 1. In addition, isolated measurements have been obtained from twenty-two patients at intervals of two days to five months after the ingestion of Priodax. These are shown in Fig. 2. In only one of the seven patients in Fig. 1 had SPI returned to its original concentration by the end of ten weeks. This value is subject to some doubt because the SPI was somewhat higher at twelve weeks. The majority had fallen by the end of thirteen weeks, but two had not reached the base line after this interval. The iodine of another patient, having followed an irregular course throughout, between the thirteenth and sixteenth weeks rose to 7.8 gamma per cent, a level sometimes found in untreated hyperthyroidism. It is obvious that this rise cannot be

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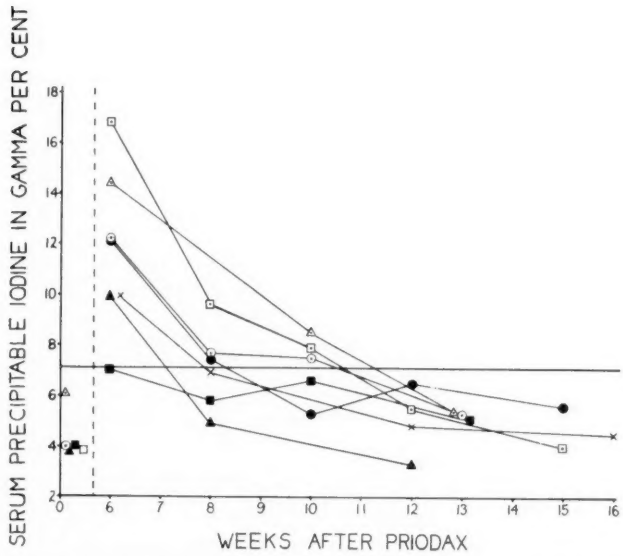

Fig. 1.—The duration of elevated serum precipitable iodine after oral ingestion of Priodax in seven patients. Values preceding administration of the drug are shown at the left of the broken line. The horizontal line indicates the upper statistical normal limit of serum precipitable iodine.

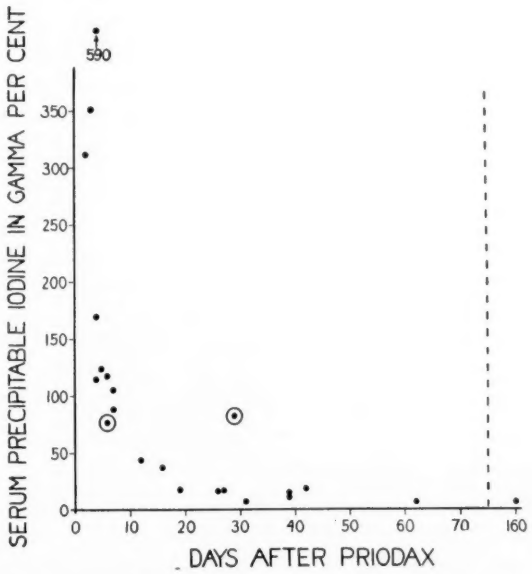

Fig. 2.—The course of serum precipitable iodine after oral administration of Priodax. The points surrounded by circles are from patients with liver disease.

attributed to Priodax. The curve of elimination of the dye has the general exponential form of other excretory curves. This is more evident in Fig. 2 in which the earlier part of the curve is visualized. When roentgen visualization of the gall bladder is made after intravenous injection of the dye, probably a greater concentration of the contaminating organic iodine compound is present

in the blood stream at one time. For example, a precipitable iodine of 990.0 gamma per cent, three times the value two and three days after oral Priodax, was found in one patient four days after intravenous administration of the dye. So gradually are the last remnants of dye excreted that it would be impossible by casual measurement or over a short interval to determine whether SPI had reached the base line. The fall between eight and ten weeks in three of the patients in Fig. 1 does not exceed 2.1 gamma per cent. It would seem advisable, therefore, to make no measurements of SPI short of twelve weeks after oral Priodax and to accept no measurement at that interval without checking it after the passage of another two weeks. One observation in Fig. 2 suggests that in the presence of advanced hepatic insufficiency elimination of dye may be greatly delayed.

Diodrast.—The elimination of the effect of Diodrast has been studied in less detail. No measurements of SPI have been made after retrograde pyelograms. In general, the serum proteins do not seem to take up Diodrast as avidly nor to adhere to it as firmly as they do to Priodax. One hour after intravenous Diodrast SPI as high as 160.0 gamma per cent was noted on one occasion. Two and one-half hours after an injection the SPI of a woman with normal renal function had risen from 6.2 to 8.6 gamma per cent. The iodine of the serum not bound by protein at that time was 746.0 gamma per cent. Four days later the SPI had fallen only to 7.7 gamma per cent, although the iodine not bound by protein had decreased to 0.3 gamma per cent. At the end of eight days (no measurements were made in the interval) SPI had returned to normal. In a second woman the SPI nine days after Diodrast was 8.2 gamma per cent, 1.8 gamma per cent above the initial value of 6.5 gamma per cent; but by the end of fourteen days it had returned to normal. A third woman suspected of hyperthyroidism had SPI's of 9.9 and 10.0 gamma per cent before and eleven days after Diodrast respectively. The SPI of two patients three weeks after Diodrast were at expected concentrations for euthyroid individuals, but no control observations had been made. All of these subjects had normal renal function. Although the observations are few, they suggest that if renal function is unimpaired, measurements of SPI two weeks after intravenous Diodrast are reliable. Whether this would hold for patients with disease of the kidneys is quite uncertain. The SPI of a woman with normal renal function one day after Diodrast was 33.0 gamma per cent. In contrast, 125.0 gamma per cent was found at the same interval in the serum of a woman with a small left kidney and calcification of the vessels of the right kidney.

Lipiodol.—The contamination from Lipiodol is peculiarly persistent and unpredictable. Presumably it must depend on the quantity retained in the body and the rate at which it is being absorbed from the deposits at the time of the analysis of serum. One patient who was under treatment for hyperthyroidism with propylthiouracil and Lugol's solution was bronchoscoped immediately after Lipiodol. Five weeks later the SPI was 196.0 gamma per cent. Fifteen months after the Lipiodol the protein-bound iodine was 11.5 gamma per cent. At this time serum cholesterol was 165 mg. per cent and the patient had signs and symp-

toms of mild hyperthyroidism. Prior to the Lipiodol, when the clinical symptoms of hyperthyroidism were somewhat more pronounced, the SPI had been 9.3 gamma per cent. In three patients SPI was above normal eleven, thirteen, and nineteen months after they had received the compound for roentgen visualization of the bronchi. These patients did have equivocal symptoms of hyperthyroidism but measurements of basal metabolic rate were unsatisfactory. The nonprecipitable iodine of the serum also was elevated in two of these subjects. In spite of the fact that no Lipiodol was visible by x-ray, one of them, after eleven months, had 6.6 gamma per cent of nonprecipitable iodine in his serum. Normally the nonprecipitable iodine does not exceed 1.0 to 2.0 gamma per cent. This indicates that the deposits of Lipiodol discharge iodine into the blood stream for an extremely long time and that a fraction of this tends to precipitate with the serum proteins. The values of 8.7, 8.0, and 10.5 gamma per cent found in these three subjects suggest that as much as 3.0 gamma per cent may be referable to contaminating material.

One patient with equivocal signs and symptoms of hyperthyroidism had a SPI of 26.0 gamma per cent ten days after the end of a two-week course of diiodoquinone. One patient with no clinical symptoms of overactivity from a goiter had a SPI of 18.4 gamma per cent three months after Pantopaque for a myelogram. These are unexpectedly high values.

SUMMARY

The iodine of Priodax, tetraiodophthalein, Diodrast, Lipiodol, and probably other organic compounds of iodine which are used for diagnostic and therapeutic purposes is included in measurements of serum precipitable iodine. They therefore invalidate the use of SPI for the estimation of the state of thyroid activity. The duration of the contamination by some of these compounds has been studied.

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EFFECTS OF PYRIBENZAMINE ON THE RESPIRATION OF MOUSE BRAIN HOMOGENATES

THEODORE F. HUBBARD, B.S., M.D., AND LEO R. GOLDBAUM, B.S., M.S.
WASHINGTON, D. C.

PYRIBENZAMINE in therapeutic doses has been shown to have an hypnotic effect in human beings and in mice.[1] In high doses it is capable of producing convulsions in laboratory animals and in man.[2] It has been shown also to be a more potent local anesthetic than procaine.[3]

It was the purpose of this investigation to observe the effects of Pyribenzamine on the metabolism of brain tissue in an attempt to elucidate the mechanism of some of the above-mentioned effects on the central nervous system. The results obtained with Pyribenzamine can be compared with those effects which have been produced by depressants,[4] analeptics,[5] and local anesthetics[6] on the in vitro respiration of brain tissue.

EXPERIMENTAL

The method of Reiner[7] was used for glucose and pyruvate oxidation, that of Utter, Wood, and Reiner[8] for anaerobic glycolysis, and that of Schneider and Potter[9] for succinic oxidase and cytochrome oxidase activity.

The brain preparations were made by homogenizing freshly removed whole brains of ten to twelve mice in cold, distilled water in a glass homogenizer. No longer than fifteen minutes elapsed from the time the animals were killed until the experiments were begun. The rate of oxygen uptake or carbon dioxide evolution was followed in Warburg manometers at 36.3° C. The contents of the various flasks are shown in Table I. After a fifteen-minute period for gassing and temperature equilibration the stopcocks were closed and readings were taken at ten-minute intervals for one hour. All determinations were made in duplicate, and the values given represent the average of two or more determinations.

RESULTS AND DISCUSSION

The effects of Pyribenzamine on the respiration of brain tissue are shown in Table I and Fig. 1. Pyribenzamine showed selective inhibition of respiration of brain homogenates. The oxidation of glucose, pyruvate, and the anaerobic glycolysis of glucose was inhibited, whereas the succinic oxidase or cytochrome oxidase systems were unaffected. A Pyribenzamine HCL concentration of 1.5 mg. per milliliter was required to produce 50 per cent inhibition of glucose and pyruvate oxidation. This concentration was without effect on anaerobic glycolysis which required 4.0 mg. per milliliter to produce 40 per cent inhibition. The inhibition of glucose oxidation by the foregoing Pyribenzamine concentrations could be overcome by the addition of 6×10^{-4}M methylene blue.

From Department of Basic Science and Department of Chemistry and Physics, Army Medical Department Research and Graduate School.
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TABLE I. EFFECTS OF PYRIBENZAMINE ON THE OXYGEN CONSUMPTION OF MOUSE BRAIN HOMOGENATES

	CONCENTRATION OF PYRIBENZAMINE HCl (MG./ML.)							
	None	0.50	0.75	1.0	1.5	2.0	3.0	4.0
(a) Glucose oxidation*	6.10	6.07	5.49	4.90	3.06	1.54	0.92	0.59
(b) Pyruvate oxidation*	8.04	7.61	--	6.01	3.46	1.62	0.81	0.24
(c) Anaerobic glycolysis of glucose†	5.60	5.59	5.56	5.66	5.55	5.09	4.18	3.42
(d) Succinate oxidation*	3.82	--	--	3.76	3.90	3.78	3.80	3.88
(e) Cytochrome oxidase*	3.34	--	--	3.35	3.25	3.45	3.40	3.39

*Microliters of O_2 per milligram of wet weight of brain tissue per hour.

†Values for anaerobic glycolysis of glucose are in microliters CO_2.

Temperature 36.3° C.; gas phase, air; 0.1 ml. of 30 per cent KOH in center well; concentrations of Pyribenzamine HCl as shown above. Contents of flasks: (a) Glucose oxidation—glucose 0.028M; hexose diphosphate · Ba 0.005M; ATP 0.0007M; DPN 0.001M; nicotinamide 0.04M; cytochrome C 0.00006M; K_2HPO_4-KH_2PO_4 buffer pH 7.4 0.04M; 0.5 ml. 1:4 mouse brain homogenate; final volume 3.0 ml. (b) Pyruvate oxidation—sodium pyruvate 0.028M in place of glucose, otherwise the same as (a). (c) Anaerobic glycolysis of glucose—temperature 36.3° C.; gas phase 95 per cent nitrogen plus 5 per cent CO_2; concentration of solution: glucose 0.028M; hexose diphosphate · Ba 0.0025M; ATP 0.0007M; DPN 0.0005M; nicotinamide 0.04M; K_2HPO_4-KH_2PO_4 buffer pH 7.4 0.01M; $NaHCO_3$ 0.048M; 0.5 ml. 1:4 mouse brain homogenate. (d) Succinate oxidation—sodium succinate 0.05M; cytochrome C 0.00017M; $CaCl_2$ 0.0004M; $AlCl_3$ 0.0004M; Na_2HPO_4-HCl buffer pH 7.4 0.03M; 1 ml. 1:4 mouse brain homogenate; final volume 3.0 ml. (e) Cytochrome oxidase—sodium ascorbate 0.0114M in place of succinate, otherwise the same as (d).

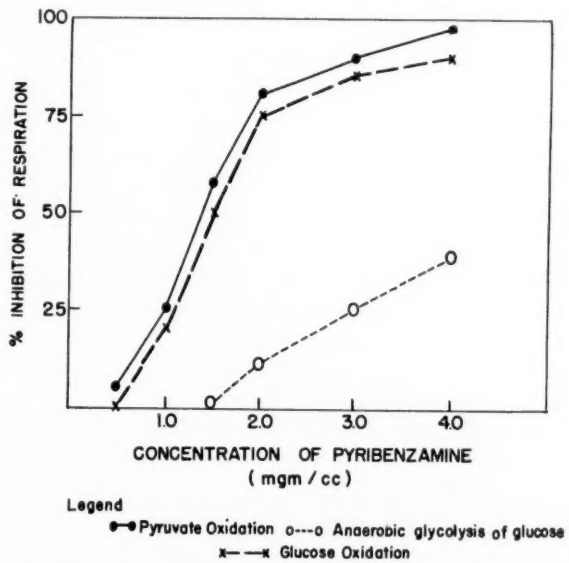

Fig. 1.—Inhibition of respiration of brain homogenates by Pyribenzamine.

Since the anaerobic glycolysis of glucose is interfered with, it might be inferred that the principal action of Pyribenzamine is to limit the supply of intermediary metabolites above the point of separation of aerobic and anaerobic carbohydrate metabolism. The inhibition of pyruvate oxidation indicates that this is not so. Leucomethylene blue can serve as a hydrogen carrier between the dehydrogenase and oxygen since it is autoxidizable in air. The ability of methylene blue to by-pass the block interposed by Pyribenzamine indicates that the dehydrogenases involved in carbohydrate metabolism are not inhibited and suggests that the block probably occurs between the pyridine nucleotides and the cytochrome system.

This inhibition is similar to that observed with the barbiturates, although the concentrations of drug required for inhibition are from four to ten times higher than those observed with barbiturates[4] and correspond to an in vivo dose of about 1 Gm. per kilogram. Since this is approximately ten times the intraperitoneal LD_{50} of Pyribenzamine, the conclusion that the inhibiting effects of this antihistaminic on the carbohydrate metabolism of brain may account for its sedative action cannot necessarily be made.

Certain parallels have been shown between the in vitro depression of brain metabolism and the anesthetic potency of some of the local anesthetic agents. The effective concentrations of Pyribenzamine are comparable to concentrations of local anesthetics which produce a similar degree of depression of in vitro brain respiration, though the latter also inhibit the oxidation and reduction of cytochrome C[5]. It is possible that the effects observed may account for the local anesthetic action of Pyribenzamine. There is no evidence to indicate that the observed effects are related to the convulsant effects caused by high doses of Pyribenzamine. The analeptic drugs which have been studied do not inhibit the in vitro respiration of brain tissue.[6]

SUMMARY

Pyribenzamine inhibits the oxidation of glucose, pyruvate, and the anaerobic glycolysis of glucose by mouse brain homogenates. The succinic oxidase and cytochrome oxidase systems are not affected by Pyribenzamine. Since the block can be by-passed by methylene blue, it is suggested that the site of inhibition is between the pyridine nucleotides and the cytochrome system. These effects may explain the hypnotic and local anesthetic effects of Pyribenzamine.

The results were obtained in in vitro studies and not after the material was injected into the animals and the brain homogenized, so that the results are only indicative of what might happen in the intact animal.

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CARDIAC LESIONS FOLLOWING VENOUS CATHETERIZATION OF THE RIGHT AURICLE AND CORONARY SINUS OF DOGS

WILLIAM G. BANFIELD, M.D.,* DONALD B. HACKEL, M.D.,† AND
WALTER T. GOODALE, M.D.‡
ARMY CHEMICAL CENTER, MD.

VENOUS catheterization of the heart, first performed by Forssmann,[1] was extensively developed and applied in man by Cournand and Ranges.[2] Although it is widely used as a diagnostic and investigative tool, reports of cardiac lesions following the procedure have been few. Johnson and co-workers[3] reported the case of a 19 pound cyanotic child with a hemoglobin of 22 grams. The catheter was introduced through the saphenous vein into the right auricle. At postmortem one month later, a well-organized thrombus was found occluding both internal iliac veins, the inferior vena cava, and the right renal vein. Another thrombus was found at the base of one of the cusps of the tricuspid valve. Cournand and associates[4] reported two cases with thermal burns in which the catheter was left in the heart over forty-eight hours. Both patients subsequently died and, at autopsy, no cardiac lesions were found. Dexter and co-workers[5] mentioned ten autopsied patients in whom there were no cardiac lesions attributable to catheterization.

In a previously reported series of twenty-eight dogs,[6] autopsied after cardiac catheterization for the determination of coronary blood flow, a high incidence of cardiac lesions was observed. However, the many variables encountered in these experiments made it impossible to evaluate the factors acting to produce the lesions. Therefore, a series of dogs was catheterized under more standardized conditions and with as little manipulation as possible to find whether lesions would still occur.

METHODS

Eighteen dogs weighing 9.1 to 28 kilograms were lightly anesthetized with Nembutal (pentobarbital sodium 25 mg. per kilogram), and the catheter was inserted gently under fluoroscopic control into the heart via the right external jugular vein. Clean but not sterile technique was employed in all but two instances in which sterile precautions were observed. A slow intravenous drip of sterile physiologic saline was used to keep the catheter free from clotting in the tip. The catheter was removed after one hour. Forty-eight hours later the dogs were sacrificed with intravenous Nembutal and autopsied immediately. No heparin was used in this experiment.

The right auricle was catheterized in seven dogs using a soft, No. 6, plastic, curved-tipped, venous catheter with multiple eyes, described by Goodale and associates.[7] In an additional five dogs the right auricle was catheterized with a No. 6, plastic, curve-tipped, venous catheter with a single terminal eye as originally used by Cournand and Ranges.[2] In two of these dogs sterile precautions were employed. The catheter was placed in the inferior vena cava and then withdrawn to the mid-right auricle where it was left with its tip pointing posteromedially. The natural course of the catheter was into the inferior

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*Present address: Department of Pathology, Yale University Medical School, New Haven, Conn.
†Present address: Department of Pathology, Cleveland City Hospital, Cleveland, Ohio.
‡Present address: Department of Medicine, Peter Bent Brigham Hospital, Boston, Mass.

vena cava and it was entered only for purposes of orientation. All auricular catheterizations were performed with great ease. There is no simpler or less traumatic cardiac catheterization procedure.

The coronary sinus was catheterized in six dogs according to the method of Goodale and associates.[6] The amount of manipulation required to place the catheter 2 to 3 cm. within the coronary sinus was of necessity greater than that for catheterizing the right auricle; however, no great difficulty was encountered in any instance. The estimated degree of difficulty judged at the time of catheterization is shown in Table II.

Forty dogs which were never catheterized were sacrificed with large intravenous doses of Nembutal after varying periods of Nembutal anesthesia. Their hearts were examined as controls.

Clotting times were determined by the Lee-White method[10] on the dogs before anesthesia, after anesthesia, and forty-eight hours later.

Electrocardiograms were taken before and during the insertion of the catheter, at the end of the experiment, and forty-eight hours later.

RESULTS

Right Auricular Catheterization.—The right auricle was catheterized in twelve dogs with extreme precautions to prevent trauma to the heart. The results are presented in Table I. It can be seen that sterile technique does not prevent the occurrence of lesions. The dogs in which a No. 6, plain-tipped,

TABLE I. CATHETERIZATION OF THE RIGHT AURICLE

DOG	BODY WT. (KG.)	HEART WT. (GM.)	ESTIMATED TRAUMA*	DESCRIPTION OF LESIONS	
				SUPERIOR VENA CAVA	RIGHT AURICLE
P-1†	15.0	122	±	1 by 2 mm. slightly raised T. Azygos ostium: pinhead sized T	Aur. Bd.: 4 by 4 mm. raised T Post. R.A.: 10 by 3 mm. T
P-3†	16.6	127	±		Aur. Bd.: pinhead sized T Post. R.A.: 5 by 3 by 3 mm. T
P-4†	9.1	74	±		
P-5†	16.0	110	±		Post. R.A. lat. to I.V.C.: 8 by 4 mm. T
P-6†	18.4	140	±		
P-7†	13.9	86	±		
P-8†	17.7	139	±		
P-16‡	22.2	180	±	6 cm. by 5 mm. T extending into R.A.	Post. R.A.: hemorrhagic. Aur. Bd.: small flat T. Base tricuspid: 1 by 1.5 cm. T; hemorrhagic
P-17‡	27.2	180	±		Below Aur. Bd.: 1 by 1 cm. flat T. C.S. ostium: small flat T. I.V.C. ostium and base of tricuspid: hemorrhagic
P-19‡	21.4	--	±	5½ cm. by 2 mm. T extending into R.A.	
P-20§	17.7	--	±	7 cm. long T extending into R.A.	Below Aur. Bd.: 3 mm. diameter T
P-21§	17.3	142	±		Lat. to Aur. Bd.: small filmy T

Aur. Bd., Thickened band of muscle above fossa ovale; C.S., coronary sinus; I.V.C., inferior vena cava; R.A., right auricle; T, thrombus.

*Estimated trauma at catheterization: minimal (±) to maximal (4+).

†Goodale-Lubin catheter used[7]; clean technique.

‡Plain-tipped catheter used; clean technique.

§Plain-tipped catheter used; sterile technique.

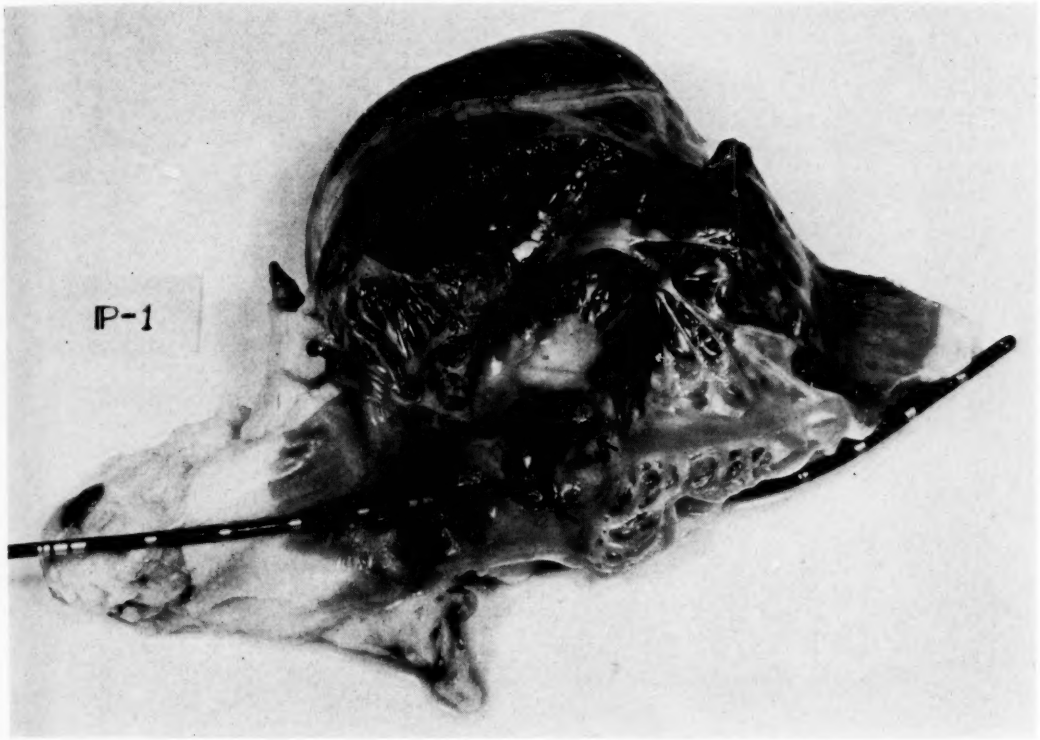

Fig. 1.—View of heart with posterior wall of right auricle turned back. The catheter is passing down the superior vena cava, through the right auricle, and out via the inferior vena cava. Arrows point to lesions. For description see text.

Cournand catheter was used developed as severe lesions as those catheterized with the Goodale-Lubin catheter. Five of the twelve dogs were free of cardiac lesions while the remaining seven showed mural thrombi at autopsy forty-eight hours later. These thrombi were on or associated with the thickened band of muscle above the fossa ovale, on the posterior surface of the right auricle, or at the base of the tricuspid valve. In two dogs, subendocardial hemorrhagic areas were seen.

Fig. 1 illustrates Dog P-1 which had one of the most severe lesions produced in this series. There was a 4 by 4 mm., rounded, smooth, raised thrombus on the lateral surface of the thickened band of muscle above the fossa ovale. Below and lateral to this on the posterior wall of the auricle was another club-shaped, 1 cm. long, 3 mm. wide, smooth, raised thrombus attached along its entire length. In the myocardium under this thrombus there was hemorrhage that could be seen through the epicardial surface of the auricle. In the superior vena cava a small thrombus was found on its medial wall and another tiny thrombus at the azygos opening. Microscopically, the thrombi were of premortem appearance, with a few fibroblasts growing into the base. In the myocardium, beneath one of these thrombi, there was a collection of polymorphonuclear leucocytes and a thin margin of degenerating muscle.

Coronary Sinus Catheterization.—In six dogs the coronary sinus was catheterized, and all of these showed some evidence of cardiac trauma. (Table II.) In the right auricle, thrombi were found in three dogs on the band of muscle above the fossa ovale. In two instances there were rough, slightly raised, granular thrombi on a large area of the posterior auricular wall. In one of these,

TABLE II. CATHETERIZATION OF THE CORONARY SINUS

DOG	BODY WT. (KG.)	HEART WT. (GM.)	ESTIMATED DIFFICULTY IN CATHETERIZATION	DESCRIPTION OF LESIONS		
				SUPERIOR VENA CAVA	CORONARY SINUS	RIGHT AURICLE
P-9	18.2	138	2+		1 by 3 mm. hemorrh. at junction of G.C.V.	
P-10	28.0	217	3+		2 small hemorrh. in epicardial fat	Lat. to Aur. Bd.: 1.5 by 3 cm. T Between C.S. and I.V.C.: small gran. T At I.V.C. ostium: 1 by 1 cm. mural T
P-11	19.5	128	1+		1 cm. long area on post. wall of hemorrh. and small mural T	
P-12	24.5	152	2+		Hemorrh. in perisinuous fat	Aur. Bd.: T and hemorrh. on surface Below Aur. Bd.: 1.5 cm. area of mural T and hemorrh.
P-13	19.1	126	1+		1 by 18 mm. T on post. wall of C.S.	Aur. Bd.: 3 mm. diameter T
P-14	23.2	172	1+	Just above R.A.: long flat T	M.C.V.: obstructing T at ostium P.V.: obstructing T at ostium and hemorrh. in myocardium about vein Between these: 2 small flat T	Aur. Bd.: 1 cm. long T extending upward

*Minimal (±) to maximal (4+).

Aur. Bd., Thickened band of muscle above fossa ovale; C.S., coronary sinus; G.C.V., great cardiac vein; hemorrh., hemorrhage; I.V.C., inferior vena cava; M.C.V., middle cardiac vein; P.V., posterior vein of left ventricle; R.A., right auricle; T, thrombus.

there was additional subendocardial hemorrhage. In a final instance there were many small subendocardial hemorrhages covering the ridge of muscle above the fossa ovale. In the coronary sinus the lesions, in general, were small. They consisted of thrombi and small hemorrhages on the posterior wall of the sinus, and in two instances of tiny hemorrhages in the perisinuous fat. In most instances, the thrombi were flat and granular and appeared to form over areas in which the endothelium had been abraded by the catheter.

Dog P-14 was of particular interest (Fig. 2). Even though the trauma of insertion was minimal, the lesions in the coronary sinus were severe. One throm-

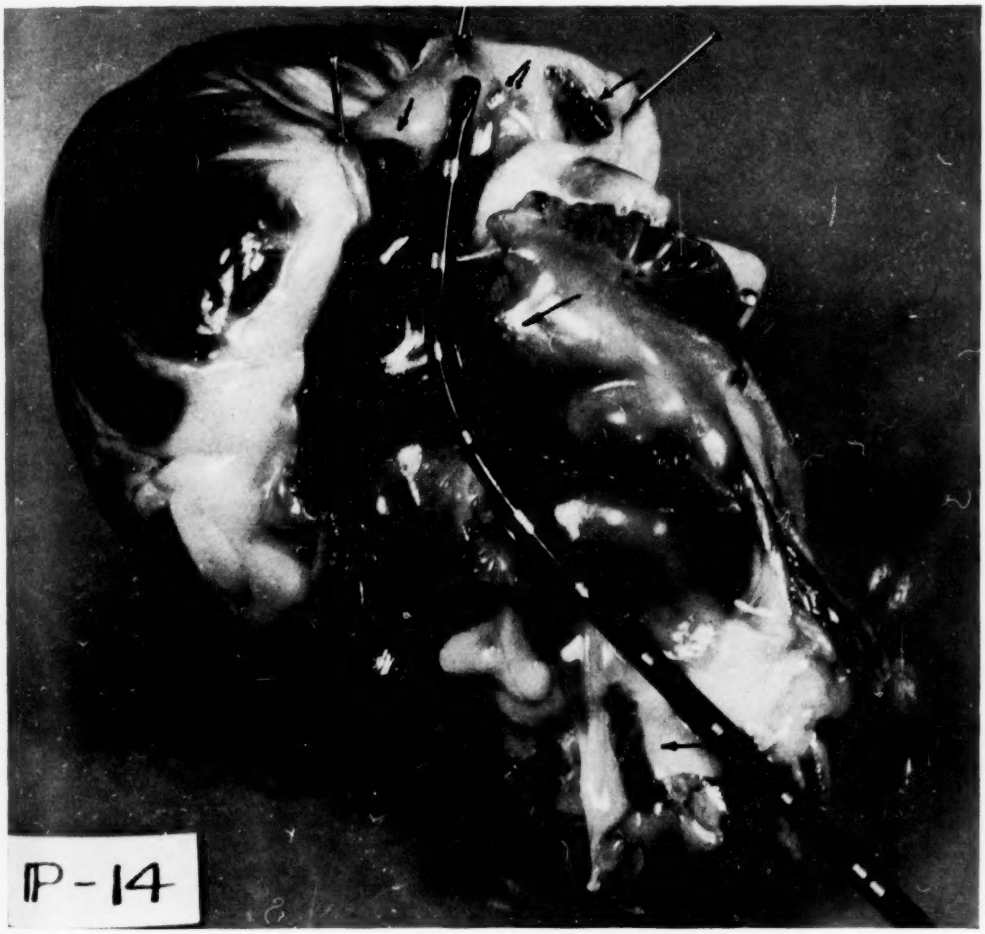

Fig. 2.—View of heart with posterior wall of the right auricle turned back and the coronary sinus opened. The catheter is passing down the superior vena cava through the right auricle, and into the coronary sinus. Arrows point to lesions. For description see text.

bus was found obstructing the middle cardiac vein and another obstructing the opening of one of the posterior veins of the left ventricle. Between these there were several small granular thrombi. About the proximal portion of the posterior vein there were many small subepicardial hemorrhages extending 1 to 2 mm. into the left ventricular myocardium, whereas the muscle around the middle cardiac vein was normal. It is interesting to note that the middle cardiac vein had an extensive anastomosis about the apex with the great cardiac vein, while the posterior vein had no grossly visible anastomosis. In the right auricle of this dog there was a pale, flat thrombus beginning on the lateral aspect of the band of muscle above the fossa ovale and extending 1 cm. above this band. On the medial surface of the superior vena cava was a long flat thrombus, and just below this was another small thrombus.

All the thrombi noted were of recent premortem formation as observed by both gross and microscopic examination.

Uncatheterized Controls.—The uncatheterized control dogs failed to show thrombi or subendocardial hemorrhages in the superior vena cava, right auricle, or coronary sinus. Occasionally, fresh subendocardial hemorrhages were observed in the left ventricle and on the mitral and aortic valves, but never in the right ventricle or tricuspid valve. A single small blood cyst, brown or black in color, was found on the medial leaflet of the tricuspid valve in several instances.

There were no statistically significant differences between the dogs' clotting times taken before, during, and after anesthesia; nor was there any correlation between the clotting time and the severity of the lesions found at autopsy.

Electrocardiograms.—The electrocardiograms taken before insertion of the catheter, one hour after insertion, and forty-eight hours later showed no significant differences in any dog in this series. There were isolated and short runs of auricular extrasystoles as the catheter entered the right auricle and isolated nodal extrasystoles were almost always produced on entering the coronary sinus. Neither ventricular extrasystoles, which occur almost invariably in catheterizing the right ventricle and pulmonary artery, nor any more serious arrhythmias were found.

DISCUSSION

The site of the lesions produced in the right auricle was relatively constant and occurred where it was expected that the catheter tip or shaft had impinged on the endocardial surfaces. Frequently lesions were found on or associated with the prominent band of muscle above the fossa ovale, which is much less pronounced in man, over which the catheter passed in all but the shallowest insertion into the right auricle. It was evident that the surface of such a structure was prone to injury from the passage of the catheter tip, and from the constant rubbing of the catheter against it during auricular systole. This band of muscle, because of its contraction during auricular systole, caused continuous movement of the catheter, which may account for the lesions on the posterior wall of the auricle and in the coronary sinus.

The superficial and transient nature of the lesions produced by cardiac catheterization is indicated in a previous report.[6] Animals in which the catheter was in place as long as five hours after very difficult catheterizations were examined a few days later and were found to have large mural thrombi, extensive areas of thrombosis, and auricular myocardial hemorrhages. However, animals treated in a similar manner but sacrificed three weeks or longer after catheterization revealed only subendocardial fibrosis in the areas in which the catheter lesions customarily occurred.

The fact that cardiac lesions following cardiac catheterization have not been found in man, except for the case reported by Johnson,[3] may be due to several factors. Thrombi may not occur because the clotting mechanism in man is less active than in the dog.[8, 9, 14] The catheter may cause less abrasion in the less active human heart, which lies within a more fixed mediastinum and beats at a much slower rate than the dog's heart under Nembutal anesthesia. Also, the band of auricular muscle above the fossa ovale in dogs, already discussed as a common site of abrasion, is less prominent in man.

A fully comparable study of catheter damage in man is impossible, since most catheterization procedures in man, fortunately, are not followed by autopsy. If an autopsy were performed weeks after catheterization, one would expect to find, judging from observations in dogs, only minimal subendocardial fibrosis of questionable significance. At least thirty human cases have been observed at autopsy following postoperative death, only a few days after cardiac catheterization, by Cournand,[11] Bing,[12] and Dexter.[13] No evidence of damage from cardiac catheterization was found in any of these hearts.

SUMMARY

The auricle and coronary sinus were catheterized in dogs as atraumatically as possible. Thrombi, and subendocardial hemorrhages, in some instances associated with small myocardial hemorrhages, were observed at autopsy forty-eight hours later. The lesions occurred at the sites where the catheter tip or shaft impinged on the endocardium. Even with the simplest procedure possible, represented by catheterization of the auricle alone, lesions, though small, were still found. The possible factors which might explain why these lesions are found in dogs but apparently not in man have been discussed.

We wish to express our appreciation to Rudy Johnson for assisting in the laboratory and to Dr. S. H. Durlacher and Dr. W. Fleischmann for their helpful advice in the conduct of the experiment and in the preparation of the manuscript.

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LABORATORY METHODS

SIMPLE SPECTROPHOTOMETRIC DETERMINATION OF ETHANOL IN BLOOD BY DIFFUSION IN AN AUTOCLAVE

GEORGE R. KINGSLEY, M.S., AND HAZEL CURRENT, B.S.
LOS ANGELES, CALIF.

THE blood ethanol methods which have been reported in the literature cannot be adapted for simple, multiple, routine, clinical determinations. For example, the method of Gettler[1] is time consuming and requires a special distillation apparatus; the method of Henry[2] requires for each determination two distillations before color development.

The present method is sufficiently accurate and simple for multiple clinical determinations. The principles of this method are accelerated diffusion of ethanol from blood into potassium dichromate, brought about in a sealed container in an autoclave, and the reduction of the dichromate as measured by means of a spectrophotometer.

MATERIALS AND METHODS

Reagents.—

Acid Potassium Dichromate: Dissolve 3.70 Gm. of potassium dichromate, C.P., in 150 ml. of water. Add slowly, stirring constantly, 280 ml. of concentrated sulfuric acid, C.P. Dilute to 500 ml. with water.

Standard Ethanol Solution: Add 2.53 ml. of absolute ethyl alcohol to 50 ml. of distilled water (at room temperature 20° C.) in a 100 ml. volumetric flask and dilute to volume.

Procedure.—Add 2 ml. of whole blood, serum, or plasma, and 2 ml. of distilled water for reagent blank, to cool (20° C.) beakers 3 cm. in diameter and 2 cm. in height. Place beakers in a standard taper 40/12 ground-glass stoppered weighing bottle 4 cm. in diameter and 5 cm. in height containing 10 ml. of acid potassium dichromate. Close glass stoppers firmly and place in an autoclave (kitchen pressure cooker with suitable gauge). Heat until a pressure of 15 pounds is obtained. Maintain this pressure for thirty minutes. Let the autoclave cool to room temperature before releasing pressure. Gently mix the dichromate solution in the weighing bottles by rotating them before removing the ground-glass lid. Remove lids of bottles as soon as possible after removing from autoclave. If the lid sticks, warm the bottle in boiling water to expand the bottle, and then loosen the lid. Remove beaker from bottle with small forceps and pour the reduced dichromate solution from the weighing bottle into a standard cuvette tube and read in spectrophotometer against a cuvette tube containing reagent blank set at 100 per cent T and 600 microns wave length.

From the Clinical Biochemistry Laboratory, Wadsworth Hospital, Veterans Administration Center.

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Standardization.—Add 0.0, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 ml. of standard ethanol solution to cool (20° C) beakers 3 cm. in diameter and 2 cm. in height. Dilute each to 2 ml. with distilled water and place in standard taper 40/12 ground-glass stoppered weighing bottles 4 cm. in diameter and 5 cm. in height which contain 10 ml. of acid potassium dichromate and continue as directed under Procedure. Readings may be plotted against concentration of ethanol of the standards or a conversion factor (K) calculated: Milligrams ethanol/100 ml. = K (2-log% T). When 2 ml. of blood were used, the value of K equaled 1350 for Coleman spectrophotometers, Models 6 and 14, using No. 6-304 B cuvettes. A linear relationship was found to exist between the concentration of ethanol and per cent transmission at 600 microns wave length.

RESULTS

Various types of containers, including the Conway diffusion cell, for autoclaving blood with potassium dichromate were investigated with regard to rapidity of diffusion without loss of ethanol or sticking of the ground-glass seal. The type of ground-glass stoppered bottle and beaker used was found to give the most rapid diffusion and it contained enough air space to keep the stopper in place during slight changes in pressure. Investigation of various pressures of the autoclave at different time intervals indicated that 15 pounds pressure for thirty minutes gave complete diffusion of ethanol from blood in the shortest time. Good recoveries were obtained, as shown in Table I, when various amounts of ethanol were added to a series of 2 ml. blood samples from five normal individuals.

Blood alcohol determinations were made on samples from patients with high concentration of blood urea and sugar to investigate the effect of possible formation of acetone bodies or other compounds which would reduce dichromate. No interference from these substances was found, as shown in

TABLE I. RECOVERIES OF ETHANOL ADDED TO 2 ML. OF OXALATED WHOLE BLOOD

NO.	ETHANOL PRESENT (MG./100 ML.)	ETHANOL ADDED (MG.)	ETHANOL RECOVERED (MG.)	ETHANOL RECOVERED (%)
1.	14	2	2.00	100.0
	14	4	3.84	96.0
	14	6	5.87	97.8
	14	8	7.97	99.6
	14	10	10.32	100.3
2.	17	4	3.84	96.0
	17	8	7.84	98.0
	17	12	11.72	97.6
3.	14	2	1.90	95.0
	14	4	3.84	96.0
	14	6	5.74	95.7
	14	8	7.62	95.3
	14	12	11.62	96.8
4.	11	1	1.00	100.0
	11	3	2.98	99.3
	11	4	4.02	100.5
	11	5	4.95	99.0
	11	6	5.86	97.7
	11	7	6.70	95.7
5.	8	2	2.04	102.0
	8	3	3.12	104.0

Table II. Blood ethanol estimates on samples from known alcoholics ranged from 100 to 480 mg. per 100 milliliters.

TABLE II. ETHANOL LEVELS OF BLOOD OF NORMAL INDIVIDUALS AND OF PATIENTS WITH HIGH BLOOD UREA NITROGEN AND GLUCOSE

NO.	UREA N (MG./100 ML.)	GLUCOSE (MG./100 ML.)	ETHANOL (MG./100 ML.)
		Normal Individuals	
1	14		8
2	10		8
3	7		0
4	8		8
5	13		16
6	13		20
7	14		20
8	10		26
9	7		20
10	8		8
11	13		20
12	17		20
		Patients	
1	132		19
2	152		10
3	74		7
4	172		7
5		346	28
6		452	14
7		622	11
8		650	12
9		336	10
10		246	14
11		326	14
12		336	7
13		290	14

SUMMARY

A simple, rapid, accurate method for simultaneous determination of ethanol in several blood samples has been described.

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PRESERVATION OF Rh AGGLUTINATING ANTISERUM WITH SODIUM AZIDE

H. C. Batson, Ph.D., Martha Jayne, B.S., and Martha Brown, B.A.
Washington, D. C.

In A previously reported study conducted in these laboratories,[1] it was found that sodium azide exhibited a marked preservative action for an Rh agglutinating serum deliberately contaminated with a mixture of viable bacteria selected as being representative of organisms commonly encountered as laboratory contaminants. Furthermore, this chemical agent was found to have no deleterious effect on the antibodies as evidenced by the maintenance of titer of preserved, sterile serums.

While these findings indicated the suitability of sodium azide as a preservative for such products, the conditions of the experiment reported were selected primarily to permit a rapid evaluation of the reagent rather than to simulate practical laboratory conditions under which preserved serums would be stored. Anti-Rh agglutinins are relatively thermolabile[2, 3, 4] and the growth of certain bacteria which might cause inactivation of the antibodies is enhanced at 37° C. Accordingly, this temperature was employed for storage of serum specimens to accelerate destruction of their agglutinating activity. In order to approximate usual laboratory storage conditions, comparable experiments were performed in which the samples were stored at refrigerator (2° to 10° C.) and room (20° to 25° C.) temperatures. These experiments were concomitant with that previously reported but necessarily were conducted over a longer period of time. The results of the extended study are presented here.

MATERIALS AND METHODS

The materials and methods employed were the same as those previously described[1] except for the temperatures of storage of serum specimens. A sterile serum containing an anti-D (anti-Rh_0) agglutinating antibody* was divided into three portions. To two portions, sodium azide was added to yield final concentrations of 0.1 per cent and 0.05 per cent respectively. The third portion was untreated. Each portion was then divided in half; one fraction was retained in the sterile condition and the other was seeded with a mixed suspension of common laboratory bacterial contaminants† so that each milliliter of serum contained approximately one million organisms. Each of the six samples was cultured, titered for Rh agglutinating activity, and dispensed into rubber-stoppered vials. Samples were stored in duplicate at room and refrigerator temperatures. At periodic intervals all samples were titered (employing successive twofold dilutions) in saline against a 2 per cent suspension of pooled Group O, D positive (Rh_0 positive) cells and tested for sterility.

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*The term agglutinating antibody as used throughout this report is considered synonymous with agglutinin, saline agglutinin, early or immune antibody, bivalent antibody, and complete antibody.

†The cultures used were *Bacillus cereus, Aerobacter cloacae, Streptococcus liquefaciens, Pseudomonas ovalis,* and *Staphylococcus epidermidis.* These were obtained from the National Institutes of Health through the courtesy of Dr. Margaret Pittman.

RESULTS

Duplicate samples were titered for anti-D agglutinating activity at frequent intervals during a period of forty-six weeks. Results from the paired samples were in close agreement (in no instance was more than a twofold difference in titer observed) and appreciable changes in titer occurred only following extended periods of storage. Accordingly, the data presented in Table I are the results of tests performed on only one set of samples following periods of storage selected as illustrative of the rate of deterioration.

TABLE I. TITERS OF ANTI-D AGGLUTINATING SERUM SPECIMENS AS AFFECTED BY BACTERIAL CONTAMINANTS, NaN_3 CONCENTRATION, TEMPERATURE, AND PERIOD OF STORAGE

SAMPLE	STORAGE TEMPERATURE	NaN_3 CONCENTRATION (%)	PERIOD OF STORAGE (WEEKS)			
			0	4	14	46
Sterile	Refrigerator (2°-10° C.)	0.10	128*	64	128	64
		0.05	128	128	256	64
		0.00	128	128	128	64
	Room (20°-25° C.)	0.10	128	64	64	4
		0.05	128	128	64	4
		0.00	128	128	64	4
Contaminated	Refrigerator (2°-10° C.)	0.10	128	128	128	64
		0.05	128	128	128	64
		0.00	128	128	128	32
	Room (20°-25° C.)	0.10	128	64	64	8
		0.05	128	64	64	8
		0.00	128	64	16	Neg.

*Reciprocals of greatest dilutions giving macroscopic agglutination of test cells.

It was found that sodium azide, in the concentrations tested, was bacteriostatic, but not bactericidal, for the test organisms at both refrigerator and room temperatures. Viable organisms were recovered from the contaminated samples at all test periods. Gram-negative bacilli, gram-positive bacilli, and streptococci were found in the untreated contaminated specimens, but the streptococcus was the only strain recovered consistently from the sodium azide-treated samples.

Refrigerated samples, either sterile or contaminated, exhibited little, if any, change in titers during the period of the study. Loss of activity at room temperature was gradual but was clearly evident at forty-six weeks. This difference in retention of titer of the samples stored at the two temperatures is indicative of the thermal instability of the Rh agglutinating antibody. The titers of treated and untreated sterile specimens at both storage temperatures were comparable at all test periods. This indicates that sodium azide in concentrations of 0.1 per cent and 0.05 per cent exerted no deleterious effect on the activity of the Rh agglutinating antiserum stored at either room or refrigerator temperature.

All of the sodium azide-treated, contaminated samples retained agglutinating activity comparable to that of the corresponding sterile samples. In sharp contrast, the unpreserved contaminated samples stored at room temperature showed a rapid loss in titer. Some loss was evident at fourteen weeks and the antibody was completely inactivated at twenty-five weeks. It is apparent that certain of the bacterial contaminants caused inactivation of the Rh agglutinating antibody. No attempt was made to identify which one (or more) of the particu-

lar organisms employed was responsible for this inactivation. It was evident, however, that sodium azide inhibited the bacterial growth and prevented the antibody inactivation resulting from this source.

SUMMARY

The use of sodium azide as a preservative for an Rh agglutinating antiserum stored at room and refrigerator temperatures was investigated. It was found that, at these temperatures, sodium azide, in concentrations of 0.1 per cent and 0.05 per cent, was bacteriostatic and had no apparent deleterious effect on the agglutinating activity of the antiserum employed.

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THE PRESERVATION OF SHEEP CELLS FOR HETEROPHILE ANTIBODY DETERMINATIONS

ALFRED S. EVANS, M.D.*
NEW HAVEN, CONN.

THE knowledge of better methods of preserving blood gained in recent years has been applied to serologic techniques requiring the use of red blood cells. For instance, it has been found that sheep red blood cells stored over a long period of time are still satisfactory for complement fixation tests.[1] However, the use of such preserved cells in heterophile antibody agglutination methods has not been established. Indeed, earlier work suggested that sheep red blood cells stored in citrate for over a week were unsatisfactory for this purpose.[2]† In the present paper mention will be made of the results of tests comparing several techniques of preservation of sheep red blood cells and the variability in titer occasioned by storage over long periods of time when these methods are employed.

EXPERIMENTAL

Comparison of Preserving Agents.—Ten milliliters of freshly drawn sheep blood were prepared by each of the following methods: (1) defibrination with glass beads; (2) heparin, 2 mg.; (3) 5 per cent sodium citrate, 2.0 ml.; (4) modified Alsever's solution,‡ 10.0 milliliters. The samples were stored at 4° C. in a refrigerator. At weekly intervals 2.0 ml. of each blood sample were withdrawn, washed three times in saline, and made up as a 1 per cent suspension. These were tested against serial doubling dilutions of the same standard serum for a period of five consecutive weeks. Fresh blood drawn weekly from the same sheep was used as a control. The results are shown in Table I, where it will be observed that the use of sodium citrate gave the most consistent results though variations of only 1 or 2 tubes in titer were seen with any of the other preserving methods. The degree of hemolysis at the end of the test period was somewhat greater with sodium citrate than with modified Alsever's solution, but it was much less than that observed with either defibrinated or heparinized blood.

Further Experiments With Sodium Citrate.—To minimize hemolysis a larger amount of sodium citrate was tested as a preservative: one sheep was bled at consecutive weekly intervals and the blood was stored in one half its volume of 5 per cent sodium citrate at 4° C. On the tenth week these ten sheep blood preparations were washed and tested against a standard serum, titer 1:640.

From the Section of Preventive Medicine, Yale University School of Medicine.

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*National Institutes of Health Postdoctorate Research Fellow, 1948-49.

†Since the completion of this study, Rappaport and Skariton[3] have reported that sheep red blood cells may be preserved for as long as two months by the addition of borax to defibrinated blood.

‡Alsever's solution: Dextrose, 2.05 Gm.; sodium citrate, 0.80 Gm.; citric acid 0.055 Gm.; sodium chloride, 0.42 Gm.; distilled water q.s. 100.0 milliliters. Sterilize by filtration and use equal parts with blood to be preserved.

TABLE I. RESULTS OF HETEROPHILE ANTIBODY TESTS USING A STANDARD SERUM AND SHEEP RED BLOOD CELLS PRESERVED BY FOUR DIFFERENT METHODS FOR A PERIOD OF FIVE WEEKS

STORAGE AGE IN WEEKS	PRESERVING METHOD USED				FRESH R.B.C. CONTROL
	SODIUM CITRATE	ALSEVER'S	HEPARIN	DEFIBRINATION	
0	640	320	640	640	640
1	640	640	640	640	640
2	640	1,280	1,280	1,280	640
3	640	640	640	640	640
4	640	640	640	640	640
5	640	640	1,280	1,280	640

Titer is expressed as reciprocal of final serum dilution.

Identical heterophile antibody titers were found with each suspension of red blood cells, indicating that this amount of citrate did not alter the susceptibility of the cells. Hemolysis was minimal in unwashed cells up to six weeks of storage but was appreciable after that time even though similar agglutinative titers were found after washing.

In another experiment, 100 ml. of sheep blood were stored in 50 ml. of 5 per cent sodium citrate at 4° C. for a period of six months. At this time it was washed and tested against nine sera of different titers from patients with infectious mononucleosis. Fresh sheep red blood cells were used as a control and all tests were done in duplicate. The results are shown in Table II, where it will be seen that in four of the sera the titer was identical with that obtained with the fresh sheep red blood cells and in the other six there was a difference of only one tube.

TABLE II. RESULTS OF HETEROPHILE ANTIBODY DETERMINATIONS ON NINE SERA USING FRESH SHEEP RED BLOOD CELLS AND SHEEP RED BLOOD CELLS PRESERVED FOR SIX MONTHS IN SODIUM CITRATE

SERUM	HETEROPHILE TITER	
	6 MO. OLD R.B.C	FRESH R.B.C.
1	80	160
2	160	160
3	160	320
4	160	320
5	160	160
6	320	320
7	320	640
8	320	640
9	640	1,280

Titer is expressed as reciprocal of final serum dilution.

Storage of Sheep Red Blood Cells as a 1 per cent Saline Suspension.—Fresh sheep red blood cells were washed three times and made up as a 1 per cent suspension in saline. This suspension was tested against the same serum when the red blood cells were fresh, and on the second, ninth, and fifteenth day of storage at 4° C. Identical titers were obtained even though hemolysis was marked on the fifteenth day.

DISCUSSION

A comparison of the use of sodium citrate, heparin, modified Alsever's solution, and defibrination as methods of preserving sheep red blood cells for heterophile antibody agglutination tests has been made. Sodium citrate appeared to

give the most consistent results and was attended by a minimum of hemolysis over a period of storage of six weeks. Heterophile titers identical to those obtained with freshly drawn sheep red blood cells were found with red blood cells preserved by this method over a period of ten weeks, and even after storage up to six months, variations in titer greater than 1 dilution were not encountered.

It is interesting to note that the presence of marked hemolysis in unwashed preparations does not necessarily make them unsuitable for agglutination tests after washing. This suggests that up to the critical point of hemolysis an individual red blood cell may show little change in its agglutinability.

SUMMARY

1. Sodium citrate appears to be a somewhat better agent for the preservation of sheep red blood cell heterophile antibody agglutination tests than modified Alsever's solution, heparin, or defibrination.

2. Sheep red blood cells preserved by sodium citrate were found to be suitable for agglutination tests after ten weeks of storage, and even after six months little alteration in the agglutinability of the red blood cells was observed.

3. The presence of hemolysis in unwashed sheep red blood cells does not necessarily make them unsuitable for heterophile agglutination tests.

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A SIMPLE MODIFICATION OF THE MIDDLEBROOK AND DUBOS HEMAGGLUTINATION TEST FOR SERUM ANTIBODIES TO PRODUCTS OF TUBERCLE BACILLI

NANCY B. SCOTT, M.A., AND DAVID T. SMITH, M.D.
DURHAM, N. C.

IN A recent paper by Middlebrook and Dubos,* a method of testing for antibodies in human sera against the products of tubercle bacilli was described. The paper deals with the technique and results of hemagglutinations employing washed sheep erythrocytes sensitized with aqueous extracts of tubercle bacilli. These sensitized red cells are thus rendered agglutinable by specific antibodies in the sera of experimental animals and of tuberculous patients.

The extract which they prepared utilized dried tubercle bacilli of the strain H37Rv grown on the surface of a liquid medium for fourteen days and then processed for several days until a clear, isotonic extract was obtained. Middlebrook and Dubos stated that material from the tubercle bacilli found in at least one sample of old tuberculin, after deglycerination, sensitized red cells to agglutinate in tuberculous antisera. They found that 1 ml. of deglycerinated old tuberculin was capable of sensitizing completely 0.025 ml. of washed, packed sheep erythrocytes so that these red cells were agglutinated in the same way and to the same titer in an anti-BCG immune serum as were red cells of the same lot sensitized by their extract of dried tubercle bacilli.

The purpose of the present paper is to describe a modification of the Middlebrook and Dubos hemagglutination test, which appears equally specific and at the same time sufficiently simple and cheap to be utilized by hospitals and institutions not equipped or staffed to prepare the original aqueous extracts or to deglycerinate commercial tuberculin.

The authors of this paper sought the aid of Lederle Laboratories in finding a suitable old tuberculin, commercially obtainable, which would meet their needs in performing a hemagglutination test for antibodies to tubercle bacilli. An old tuberculin of four times standard strength was supplied by their laboratories to be used for this purpose.

The hemolyzing effect of the glycerine in old tuberculin upon the red cells was eliminated by dilution of the 4× O.T., using buffered isotonic saline as the diluent. A hemolytic test with serial dilutions of O.T. was performed on normal, washed sheep erythrocytes and a dilution of 1:8 and above rendered the tuberculin nonhemolytic to the red cells.

In another series of titrations of tuberculin against tuberculous antisera, it was found that there was complete sensitization of red cells until a dilution of more than 1:20 was reached.

From the Department of Bacteriology, Duke University School of Medicine.
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*Middlebrook, Gardner, and Dubos, R. J.: Specific Serum Agglutination of Erythrocytes Sensitized With Extracts of Tubercle Bacilli, J. Exper. Med. 88: 521-528, 1948.

A dilution of 1:15 of tuberculin in buffered, isotonic saline was arbitrarily chosen for the sensitization of red cells. Although the stock tuberculin remains stable for a long period of time at 4° C., the dilutions for the test were freshly prepared each day. A concentration of 0.1 ml. packed, washed sheep erythrocytes per 6.0 ml. diluted old tuberculin was used. It was found that a concentration of red cells greater than 0.1 ml. per 4.0 ml. diluted tuberculin did not give complete sensitization of the red cells and resulted in lower titers when these cells were agglutinated in tuberculous antisera of previously determined antibody titers.

METHODS AND MATERIALS

Preparation of Washed, Packed Sheep Erythrocytes.—Sheep red cells stored in Alsever's solution* were centrifuged for ten minutes at 1,800 r.p.m., and the supernatant was removed with a pipette. These red cells were then washed three times with 6 vol. of isotonic saline solution. Following the last washing, the cells were centrifuged for twenty minutes at 2,000 r.p.m., and as much of the supernatant as possible was removed with a small pipette. These cells were used in the test as "packed washed cells."

Preparation of Antigen.—Old tuberculin of four times standard strength (Lederle) was diluted 1 to 15 in buffered isotonic saline, using 0.4 ml. tuberculin to 5.6 ml. buffered saline. To this, 0.1 ml. packed, washed red cells was added and incubated for two hours in a 37° C. water bath. The mixture was thoroughly shaken at approximately fifteen-minute intervals. This cell suspension was removed from the water bath and centrifuged at low speed (approximately 1,000 r.p.m. for six minutes). A low speed is desired to avoid a hard button of red cells which is difficult to resuspend without hemolyzing some of the red cells. The cells were washed three times with 6 ml. volumes of buffered saline and finally resuspended in 50 ml. buffered saline, to be used as antigen in hemagglutination tests. This antigen may be stored for three days at 4° C. temperature.

Absorption of Patient's Serum.—One milliliter of patient's serum was added to 1.0 ml. buffered saline; the solution was inactivated at 65° C. for three minutes, cooled to room temperature, and 0.2 ml. of untreated, packed, washed sheep erythrocytes were added; the mixture was allowed to stand at room temperature for at least twenty minutes. The cells were removed from the serum by centrifugation at 2,500 r.p.m. for seven minutes and discarded. Another 0.2 ml. volume of untreated, packed red cells was added to the serum and the mixture was allowed to stand for another period of twenty minutes at room temperature, after which the cells were removed by centrifugation. This absorption of the patient's serum with normal red cells removes the heterophil antibodies which are capable of agglutinating normal red cells.

Absorbed serum may be stored indefinitely in a deep freeze without alteration of antibody titer to sensitized red cells.

Hemagglutination Test.—Using standard size Wassermann tubes and racks, tests were set up with absorbed serum in twofold serial dilutions. Four-tenths milliliter of the absorbed serum was added to the first and second tubes. Serial dilutions were made from the second tube through eight tubes, with equal volumes of buffered physiologic saline. To tubes containing 0.4 ml. of serum dilution there was added 0.4 ml. of 0.2 per cent suspension of sensitized red cells. Two control tubes were used—one tube containing 0.4 ml. of saline and 0.4 ml. of sensitized red cell suspension being used as antigen, and another tube containing 0.4 ml. of 1:2 dilution absorbed serum and 0.4 ml. of a 0.2 per cent suspension of untreated, washed, sheep erythrocytes. A known positive and a known negative serum were

*Each 100 ml. of Alsever's solution contains: dextrose, 2.05 Gm.; sodium citrate, 0.8 Gm.; and sodium chloride, 0.42 Gm. The original Alsever formula has been recently modified by the addition of 0.055 Gm. of citric acid per 100 ml. The finally sterilized solution has a pH of 6.1 and shows very slight or no evidence of caramelization. (Bukantz, S. C., Rein, C. R., and Kent, J. R.: Studies in Complement Fixation. II. Preservation of Sheep's Blood in Citrate Dextrose Mixtures (Modified Alsever's Solution) for Use in the Complement Fixation Reaction. J. LAB. & CLIN. MED. 31: 394, 1946.)

included each time unknown sera were tested. The negative sera were obtained from individuals with negative tuberculin skin tests.

The tubes were shaken and then incubated in a 37° C. water bath for three hours. Upon removal from the water bath, tubes were reshaken and left at room temperature overnight, to be read the following morning.

Tests were read in a manner similar to the usual type of red cell agglutination.

RESULTS

A series of fifty sera of tuberculous patients were tested and thirty-eight of them were found to have agglutinins to products of tubercle bacilli at a serum dilution of 1:2 or greater. Forty medical students and nurses who had received BCG four months previously were tested, and of this number thirty showed positive results in hemagglutinations for antibodies to tubercle bacilli. In a group of sera of fifteen persons having a negative tuberculin skin test, all hemagglutinations for antibodies to tubercle bacilli were negative.

The clinical significance remains to be determined and is under investigation in a larger series of patients.

All the original tests were performed with a single lot of old tuberculin obtained from the Lederle Laboratories. To determine if random lots of this 4× old tuberculin would prove to be equally reliable, we obtained samples from six fresh lots. The original lot and these six lots were used concurrently to sensitize red cells, which were in turn used as seven separate antigens to test a total of ten positive sera and ten negative sera. A sample protocol is seen in Table I. Total results are found in Table II. These indicate that there is no significant difference in the lots of old tuberculin in regard to power to sensitize red cells, slight differences being within normal limits of error in dilution of sera and performance of tests.

TABLE I. SAMPLE PROTOCOL SHOWING RESULTS OF HEMAGGLUTINATIONS OF SEVEN ANTIGENS WITH SERUM F

ANTIGEN	SERUM DILUTION							
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
1	+	+	+	±				
2	+	+	+	+	±			
3	+	+	+	±				
4	+	+	+	+				
5	+	+	+	+				
6	+	+	+	±				
7	+	+	+	+				

DISCUSSION

It is hoped that the hemagglutination procedure described will enable workers to test a large number of sera from many patients with tuberculous disease in its various stages and forms, as well as those patients in whom a definite diagnosis of tuberculosis has been impossible to establish. It will be equally interesting to add to the data tests on individuals not having tuberculosis, in an attempt to determine whether false positive results are obtained, and if so, under what circumstances they may be expected.

TABLE II. RESULTS OF HEMAGGLUTINATIONS IN TEN POSITIVE AND TEN NEGATIVE SERA WITH SEVEN ANTIGENS, EXPRESSED IN GREATEST SERUM DILUTION GIVING POSITIVE REACTION

ANTIGEN	POSITIVE SERA										NEGATIVE SERA									
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
1	1:32	1:64	1:8	1:2	1:2	1:8	1:2	1:64	1:8	1:4	0	0	0	0	0	0	0	0	0	0
2	1:64	1:64	1:8	1:2	1:2	1:16	1:2	1:64	1:16	1:8	0	0	0	0	0	0	0	0	0	0
3	1:64	1:64	1:8	1:2	1:2	1:8	1:2	1:64	1:16	1:4	0	0	0	0	0	0	0	0	0	0
4	1:64	1:64	1:16	1:2	1:2	1:16	1:2	1:64	1:16	1:8	0	0	0	0	0	0	0	0	0	0
5	1:64	1:64	1:16	1:2	1:2	1:16	1:2	1:64	1:8	1:4	0	0	0	0	0	0	0	0	0	0
6	1:64	1:64	1:16	1:2	1:2	1:8	1:2	1:64	1:16	1:8	0	0	0	0	0	0	0	0	0	0
7	1:64	1:64	1:8	1:2	1:2	1:16	1:2	1:64	1:8	1:4	0	0	0	0	0	0	0	0	0	0

SUMMARY

A hemagglutination test has been described for the demonstration of antibodies in human sera to the products of tubercle bacilli. It is a test which can be performed in a half day's time, to be read the following morning. Old tuberculin used in sensitizing the red cells to be used as an antigen is commercially available and sufficiently stable that it may be stored for a relatively long period of time at a temperature of 4° C.

The data available at present indicate that the test is specific for antibodies of tubercle bacilli found in human antisera.

A LIQUID MEDIUM COMPOSED OF DEHYDRATED INGREDIENTS FOR CULTURE OF ENDAMOEBA HISTOLYTICA ASSOCIATED WITH A SINGLE BACTERIUM

EDER L. HANSEN, PH.D.
SAN FRANCISCO, CALIF.

THE media used initially for cultivation of *Endamoeba histolytica* were diphasic in character, consisting of a rich coagulated slanted phase overlaid by a nutritionally poorer liquid phase, and included various combinations of egg, liver, serum, and albumin. These media have served for the isolation of parasitic amebas from infected fecal material, but, for the purpose of large-scale testing of potential amebacidal agents, they suffered from the defects of tedious preparative procedures, difficulty of standardization of ingredients, and most important of all, a variable and unpredictable absorption of test material from the liquid phase.

A liquid medium composed of standardized dry ingredients was, therefore, designed, in which the only solid material is rice powder. In this medium *E. histolytica* associated with a monobacterial flora has been carried continuously since June, 1945, and more than 300 separate tests on potential amebacidal agents have been carried out. The medium also has been successfully used by other collaborators during the course of our general study. It has proved of value in devising a simple method to test whether or not an apparently amebacidal agent is effective indirectly through inhibition of growth of the associated bacteria.[1] This medium has been used, conveniently, to produce large numbers of trophozoites and has been used for studies of the correlation between the growth cycle of amebas and the changing oxidation reduction potentials. It also has been used in excystment studies.[2]

MATERIALS AND METHODS

The culture used for the basic studies was strain 103, which had been started by microisolation by Dr. Rees in November, 1941. The amebas were associated with the nonpathogenic gram-negative bacillus, organism *t*, which can be considered as microaerophilic.

Another culture of amebas, N.R.S. strain, (Dobell) associated with a streptobacillus was established by Dr. Rees in egg slant medium. It was transferred to liver-proteose-peptone medium in February, 1949, and has since maintained vigorous growth. However, still another culture isolated by Dr. Rees, strain 107, associated with *Clostridium perfringens* would not survive in this medium after transfer from the egg slant medium.

The liver-proteose-peptone medium has been briefly referred to in previous publications[3-8] and was variously designated as "liquid liver" or "liver-proteose-peptone medium." The present report describes its preparation and use in sufficient detail to permit utilization by others.

The medium was developed during the course of investigation of liquid media in general. It was found that cultures would survive for only one transfer in the serum-saline medium

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recommended for use in drug testing by Laidlaw and co-workers[9]; nor could the amebas be maintained in a liver infusion medium or in Bacto-Thioglycollate Medium, No. B237. However, in 1945 a satisfactory medium was prepared which was essentially an infusion of coagulated, fresh, whole egg.[10] The medium was not used extensively for the testing of new agents, nor were other egg infusion media[11, 12] tried because the simpler formula reported here was developed and proved highly successful.

The advantages of the present formulation are that the medium can be prepared rapidly from standardized dry ingredients that are readily available, and, since it contains no serum, sterilization can be carried out by autoclaving. The absence of the solid slant and the relatively low growth of bacteria greatly facilitated observations of the numbers and morphology of trophozoites. The presence of starch granules did not unduly complicate observation of the ameba with the microscope.

COMPOSITION	GRAMS PER 100 ML. MEDIUM
Proteose-peptone, Difco No. B120	2.0
Liver, Wilson's Fraction L, enzymatic digest*	0.3
l-cysteine hydrochloride	0.1
Methionine	0.02
Cholesterol	0.004
Rice powder, Difco No. B146	0.4
NaCl	0.5
$Na_2HPO_4 \cdot 7H_2O$	1.0
KH_2PO_4	0.1

Preparation of Medium.—The last three ingredients in the foregoing table were dissolved in the concentrations indicated to form a stock buffered saline which was made and stored in lots of 4 liters each. The medium itself was prepared periodically in small amounts, usually about 500 ml., as needed, by dissolving the other ingredients in the stock solution. The proteose-peptone, liver digest, cysteine, and methionine were added first and dissolved with occasional shaking in about ten minutes. Following this, the cholesterol was dispersed by mild agitation, completing the preparation. The rice powder was added separately to the test tubes after dispensing and sterilization.

The liquid volume of 4.5 ml. was conveniently dispensed by using an Improved Cornwall Syringe No. 1250 and an Automatic Double Valve No. 470V.†‡ Sterilization was carried out by autoclaving for ten minutes at 123° C. and 15 pounds pressure. The medium appears as a clear, light-brown liquid. A slight sediment of minute amorphous particles and cholesterol crystals sometimes formed on standing. Rice powder was sterilized with dry heat at 100° C. for one hour on at least three occasions. Approximately 5 mg. of it were added to each tube of the medium. The rice powder was conveniently stored, as suggested by Dr. Rees, in a small flask sealed to a dispensing hood, the required amount being released by tapping the container.

Constituents of Medium.—Since the medium was designed solely for the purpose of obtaining abundant growth of the trophozoites, no attempt was made to ascertain the minimum amounts of the several ingredients. However, during development of the medium it had been noted that addition of only two-thirds of the specified quantities of dry ingredients to the saline stock resulted in poor growth. A medium containing 0.5 per cent proteose peptone and 0.1 per cent liver, even with the addition of 0.3 per cent sodium thioglycollate, supported growth during only three transfers.

Addition of serum or the infusion of coagulated whole egg did not improve growth. When erythrocytes were added, they were readily ingested by the trophozoites, but there appeared to be no improvement in growth of amebas.

Cholesterol: Cholesterol (Pfanstiehl Chemical Co.) was included in the medium on the rather empirical basis that it had been found to be a requirement for amebic growth in an

*Kindly supplied through the courtesy of Dr. David Klein, Wilson Laboratories, Chicago, Ill.

†Suppliers: Becton Dickinson, Rutherford, N. J.

‡It is a pleasure to record the visit of Dr. Y. T. Chang, one of whose many suggestions was the use of this rapid dispensing method.

entirely different type of medium.[13] However, one series of cultures was maintained through sixteen transfers in medium from which cholesterol had been omitted. Dispersion of the cholesterol from chloroform and alcohol solutions and from aqueous solution containing sodium glycocholate or bile salts was tried, and this as well as the substitution of coprosterol caused no improvement in growth. A soluble salt, cholesterol sodium sulfate was recommended by Dr. Rees and prepared by Dr. Gordon Alles and associates and more recently supplied by Hynson, Westcott and Dunning, Inc. This was used satisfactorily in several tests, but was not always included.

Methionine: Methionine (Eli Lilly & Company) was included because it had been found to be stimulatory when added to cultures in both the egg slant medium and egg infusion media. A recent trial, using only a single series of subcultures, in media without methionine suggested that it was not essential, but it was deemed inadvisable to change the formulation in view of the usefulness of the medium as originally developed.

l-Cysteine Hydrochloride: l-Cysteine hydrochloride (Pfanstiehl Chemical Co.) is essential, and in its absence trophozoites did not survive. It was added in order to produce some degree of reduction in the medium. It cannot be replaced by the oxidized form, cystine. Populations in this medium were not improved by rendering the conditions more anaerobic either by sealing the surface with melted petrolatum or by holding the cotton-stoppered tubes in nitrogen. This is an interesting contrast to the necessity for imposed anaerobiosis found by Zuckerman and Meleney[14] with their fluid encystment medium.

Liver Extract: Various types of liver preparations have been used in the cultivation of *E. histolytica*[15, 16] as indeed for many other organisms. Wilson's Fraction L, enzymatic digest, was chosen on the advice of Dr. Louis Greenberg who had used this product in nutritional studies in mammals.

Proteose Peptone: Proteose peptone is a dehydrated acid digest of protein. It was selected because of its high content of proteoses relative to peptones[17] to provide the nitrogenous compounds of high molecular weight that may be needed by the amebas. Other peptone products were not tried.

Rice Powder: Rice powder was essential in this medium for cultures associated with both organism *t* and the streptobacillus. In the presence of high populations of trophozoites there was a characteristic disintegration of the rice powder masses into component granules of approximately 5 μ size. Large numbers of these granules were ingested by the trophozoites, particularly by those amebas associated with organism *t*.

It is appreciated that this rice produce is not composed of pure starch.[14] Determinations of the protein nitrogen were very kindly communicated to us by Dr. De Lamater and associates, University of Southern California, These indicate that the nitrogen content was considerably higher than that found in a pure rice starch product. However, several attempts made during the past years to substitute pure rice starch for the rice powder were unsuccessful, the population being reduced to less than one-half that of the control. These observations were made on the third successive transfer, by which time the carry over of rice powder became practically exhausted.

Since the rice powder cannot be autoclaved in the medium or subjected to temperatures much in excess of 100° C. during dry sterilization, it is a potential source of contamination. The medium therefore usually was incubated overnight following the addition of starch. However, it was frequently noticed that addition of the starch just previous to or immediately after inoculation resulted in better growth of both organism *t* and the ameba.

Addition of glucose to the medium in amounts ranging from 1.0 to 0.1 per cent greatly improved the growth of organism *t*, but survival of the trophozoites was prevented by the high acidity that developed, the pH falling to 4.8. Other attempts to substitute other carbohydrates for powdered rice have been referred to previously.[7]

Buffer: The composition of the saline was developed on the advice of Dr. Gordon Alles, with the idea of providing considerable buffer capacity against the acidity produced by organism *t*. The pH of the saline was 7.32 (range 7.22 to 7.42). The initial pH of the medium was 6.64 to 6.95 and during incubation of the culture over periods of three to nine days it did not fall below a pH of 6.4. The salts used were of reagent quality and no attempt was made to consider the effect of trace minerals.

Effect of Storage.—The medium was so easily prepared that quantities sufficient only for a period of one to two weeks were made up at one time. A preliminary investigation on ten series of cultures showed that the medium was most satisfactory when freshly prepared. After storage of the medium for a period of five to twelve days, the populations in seventy-two-hour cultures were variable, and frequently only one-tenth of the expected maximum number of trophozoites was obtained. After fourteen days the medium was much less satisfactory, and when used on the seventeenth or eighteenth day it resulted in death of amebas in some of the cultures. The deterioration was partly reduced by storage at 3° C. The medium became slightly darker on standing. There was no change in pH. The latter deterioration was not solely due to oxidation because this occurred very soon after preparation. Limited trials (three successive transfers of three series of cultures) indicated that media stored for sixteen days at room temperature (approximately 24° C.) and then autoclaved were as satisfactory as freshly prepared media.

RESULTS

Population Cycle.—The population reached its peak, 500,000 trophozoites per tube, in forty-eight hours and then slowly declined over a further four to nine days. The usual inoculum consisted of approximately 150,000 trophozoites contained in a volume of 0.5 ml. taken from the sediment of the parent culture.

Organism *t* passed through an elongated vegetative phase during the first four hours, and then terminal "endospore-like" bodies began to form. Later, only free "spores" were seen, and these became more numerous as the age of the culture increased and were much more numerous than in cultures in the biphasic egg-slope medium.

Use of Medium in Testing Materials for Amebacidal Action.—Stocks of trophozoites were maintained for the tests by making parallel subcultures at intervals of two days. Cultures for the tests were inoculated from a pool of sediments, ½ ml. of the uniformly dispersed pool being used for each inoculum. The test agent was added immediately after inoculation, and the fate of the cultures was determined by examination and subcultures at the end of forty-eight hours of incubation.

More than 300 chemicals have been tested in this medium for possible amebacidal action. These included the conventional amebacides[18] as well as plant extracts, antibiotics, and synthetic agents. The synthetic agents tested included phenyl arsonic acids, arsenoxides, hetero-cyclic compounds including halogenated hydroxy quinolines, and acridines, substituted ethylene diamines, other aromatics including substances related to Benadryl, isothioureas, aliphatic amines, nitrites, quaternary ammonium compounds, and lactones. Only those agents showing activity comparable to that of emetine were considered for further study in vivo, and these have been referred to in other publications from this laboratory.

The dilutions found effective in this medium were compared with those showing amebacidal activity in the diphasic medium.[5] Contrary to our expectations, the agents were not uniformly more effective in the liquid medium. Rather, the level of activity relative to that in the egg-slant medium depended upon the nature of the agent. Emetine, as the standard for comparisons, was ten to twenty times more active in the liquid medium than in the egg-slant

medium. A series of substituted aliphatic amines were ten to forty times more effective, and some acridine derivatives were two to ten times as effective. On the other hand, the arsenoxides showed somewhat less activity, being apparently partly antagonized by the high level of cysteine in the medium.

There was some indication that less variability in end point could be expected in tests in this medium compared with the egg-slant medium. Tests set up in duplicate gave the same end point in parallel tests. However, a fourfold difference in end points may be obtained in tests set up on different occasions.

It was noted that the activity of emetine hydrochloride appeared to be much less in this medium than reported by Laidlaw and associates[9] in liquid media. It is suggested that this was due to the relatively greater acidity of the liver-proteose-peptone medium. The range of amebacidal activity of emetine hydrochloride was from 1/100,000 to 1/400,000.

SUMMARY

An essentially liquid medium prepared with readily available dry ingredients is described for the cultivation of *E. histolytica* associated with a monobacterial flora. The use of the medium is illustrated by application to the in vitro testing of the amebacidal action of new agents.

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A METHOD FOR SECURING UNIFORMLY MIXED POURED PETRI PLATES BY MECHANICAL MEANS

LEON BUCHBINDER, PH.D., AND ELIZABETH DILLON
NEW YORK, N. Y.

ONE of the most physically tiresome procedures in a laboratory that uses inoculated poured Petri plates in great numbers is the rotation of these plates by hand to secure even distribution of inoculum. Fatigue of the hands which follows an hour or more of pouring in this manner is frequently accompanied by unsatisfactory rotation and uneven distribution of resultant colonies.

A method whereby the rotation of Petri plates is mechanized and thus the process of pouring plates accelerated and the mixing of inoculum made more uniform was devised in this laboratory several years ago. It has been in use since then and has resulted in the pouring of countless thousands of uniformly mixed plates. The method consists of the use of a rotating platform, in this instance a Boerner rotator* although other similar rotators may be used. A typical operation in our Milk Laboratory is as follows. From one to nine plates, usually six, first are inoculated with diluted material to be tested and then are placed on the rotating platform. The switch is turned on. While rotation is taking place, each Petri dish cover is lifted in turn and the agar is poured in. The machine is turned off either when by visual inspection the mixture is seen to be homogeneous or after a stated time interval. The degree of shaking of the rotator can be adjusted so that splashing of the contents on the lid is prevented. Although the apparatus has a timing device, it has not been used for this work.

From the Bureau of Laboratories, New York City Department of Health.
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*A. H. Thomas Co., Philadelphia, Pa.

A NEW VITAL STAIN FOR MALIGNANT CELLS IN VAGINAL SECRETIONS

LOCKE L. MACKENZIE, M.D., AND DOROTHEA FULLER, B.S., M.A.
NEW YORK, N. Y.

NO STAIN exists at present which will differentiate normal cells from malignant cells in vaginal secretions. The purpose of this work is to describe a rapid differential stain for cancer cells in vaginal smears.

In 1948 Strauss, Cheronis, and Strauss[1] reported a differential reduction of triphenyl tetrazolium chloride by carcinomatous tissue as compared with normal tissue.

Tetrazolium salts result from the oxidation of colored ring compounds called formazans. This oxidation is usually brought about by the action of lead tetracetate under anhydrous conditions. The salts are water soluble and colorless. Their solutions, in contact with certain reductases in living tissues, are reduced again to colored formazans. They were first prepared by Pechman and Runge[2] in 1894, then investigated by Kuhn and Jerchel.[3] Lakon[4] in 1942 used them to test the viability of seeds. Dutcher[5] later called attention to their usefulness in connection with Lakon's work. Since then other investigators, Cottrell,[6] Mattson, Jensen, and Dutcher,[7] Porter, Durrell, and Romm,[8] and Waugh,[9] have confirmed Lakon's work and indicated that triphenyl tetrazolium chloride salts may be useful to detect differences in viability of tissues, as they are reagents capable of reacting with enzymes in the chain of glycolytic fermentation.

In 1943 Warburg and Christian[10] showed that the plasma of animals with large sarcomata contained an increased quantity of zymohexase. They postulated that, in contradistinction to normal mammalian cells, the tumor cell obtains energy from glycolysis even in the presence of available oxygen. It appeared to Strauss, Cheronis, and Strauss[1] that neoplastic cells should therefore reduce tetrazolium salts at a faster rate than normal cells. These investigators took segments of malignant tissue and immersed them in 1 per cent aqueous triphenyl tetrazolium chloride at room temperature (37° C.) for twenty minutes. They found that the carcinomatous tissue turned a ruby red while normal tissue remained unstained. When these tissues were examined microscopically it was found that a diffuse red pigment was distributed both within the cancer cells and within the stroma.

In vivo, a gauze pack saturated with this salt and placed in contact with a suspected ulcer of the tongue in one instance and with an ulcer of the anus in another instance, turned typically red; pathologic sections of these lesions disclosed carcinoma.

From the Department of Obstetrics and Gynecology, the Post-Graduate Medical School, University-Bellevue Medical Center, New York University.

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Because of these interesting observations, an attempt was made to utilize this dye as a differential intravital stain in vaginal smears and cervical scrapings. Vaginal secretions were obtained in the usual manner using a Papanicolaou pipette.[11] Cervical scrapings were made with a variety of instruments. The material in each instance was placed on a petrolatum ringed slide containing 1 per cent aqueous 2, 3,5 triphenyl tetrazolium chloride, mixed, and covered with a cover slip. As a control, slides also were made in the usual fashion, fixed in ether-alcohol, and stained by Papanicolaou's[11] technique.

Results of these tests were erratic and inconclusive when the slides were left at room temperature and examined in twenty, forty, and sixty minutes. However, if they were placed on a warming table at 37° C. for twenty minutes in the light, and if Ringer's solution was used instead of water as a diluent for the triphenyl tetrazolium chloride, the results were more accurate and clear cut.

Eighty-four cases were studied by this method. These comprised instances of chronic cervicitis, leucoplakia, cervical polypi, endocervicitis, vaginitis, endometrial hyperplasia, squamous metaplasia of the cervical glands, adenocarcinoma, and squamous carcinoma.

In all instances in which carcinoma was present, cells loaded with large orange or red globules were noted. The particles were usually round or roughly polygonal. As many as fifty or sixty might be present in the cell. There was a tendency for them to be arranged around the nucleus, often in a horseshoe pattern. Such bodies also were noted outside the cell at times. Occasionally there seemed to be a tendency for the bodies to coalesce into a much larger, irregular red globule. Cells from the deeper layers of the squamous epithelium were more often filled with these red bodies.

In certain nonmalignant conditions cells also were seen to contain red globules, but these appeared much finer and smaller than those seen in carcinoma. They also tended to be more widespread, often covering large numbers of cells as well as areas in which there were no cells. Here the pigment resembled a precipitate more than a cell inclusion. Many bacteria took a brilliant red stain. If a piece of cervical gland was scraped off, it generally contained certain cells in which were many of the fine red globules, and often a diffuse red pigment was noted all over the gland. However, in most of the nonmalignant conditions, nothing turned red.

One disadvantage noted in this stain is the fact that it may fade within twenty-four hours, and, up to the present time, no way has been discovered to make the red color permanent. If the slide was fixed in ether, alcohol-ether, methyl alcohol, acidified alcohol-ether, or Formalin alcohol-ether, no red bodies remained even in cases of carcinoma. Drying produced precipitation and marked distortion of the cells. The color faded rapidly in Paragon mounting medium.

It would appear from this preliminary work that tetrazolium chloride will stain vaginal secretion in fresh preparations. If the patient has a malignant lesion of the genitalia, a more or less typical configuration of red globules appears in certain cells. These are seen within a few minutes and have not been observed in a small series of nonmalignant conditions. The stain is not, how-

ever, entirely differential, since somewhat similar red globules also appear in nonmalignant conditions. It may be possible in the future to be able to distinguish between a "malignant reaction" and a "nonmalignant reaction." Further work is needed to attempt to fix the color permanently in the cell. In the meantime, the stain provides a rapid screening technique and might prove to be of definite value in cytologic work.

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AN APPARATUS FOR EXERCISING A PATIENT IN THE RECUMBENT POSITION

GERALD J. BRONFIN, M.D., SIDNEY H. DRESSLER, M.D., AND A. RAVIN, M.D.
DENVER, COLO.

AN APPARATUS has been designed in order to exercise patients who are being studied by the technique of right heart catheterization, the latter procedure necessitating the supine position while the patient is being exercised. The work is done by the lower extremities and the motion used is comfortable to the patient. Considering the wide range available in stroke distance and weight lifted, the apparatus is relatively simple in construction and comparatively inexpensive to build.

The apparatus is made of aluminum and is bolted in its proper position to the end of the table. The patient's feet are strapped into the pedals (*A*) (Fig. 1) and the work consists of raising weights (*B*) by pushing the pedals to the machine. The pedals are attached to three-fourth inch square aluminum bars (*C*), seventeen inches long, by means of a spring hinge (*D*). A leather washer (*E*) cushions the shock encountered at the end of the stroke. The hinge permits the pedal to tilt as the foot is drawn back (Fig. 2) and is of great importance in facilitating motion and providing comfort while exercising. The aluminum bar moves between four brass spools (*F*). Attached to the end of the bar, opposite the pedal, is an airplane strut steel wire one-eighth inch in diameter. This goes through a vertical hole at the end of the bar and is knotted on top to hold it in place. A set screw (*G*) at the end of the bar can also be tightened to fix the wire. From its set position, the wire travels underneath the bar to the brass pulley (*H*), then down to the pulley *I*, and back up through a hole in the bottom plate of the apparatus. It is knotted above the plate at this point to fix it. Pulley *I* is used because it was found that by decreasing the distance through which the weights moved by half and doubling the weight, the swinging motion of the weights was markedly decreased. The bar has a series of horizontal holes in it. A small iron pin (*J*) fits these holes and is held in place by a short section of snugly fitting rubber tubing on each side. The small pieces of rubber on the pin strike the metal plates (*K*) and prevent a noisy metal-to-metal clash. By putting the pin in the first hole in the bar, the pedals are kept tightly against the apparatus when not in use (Fig. 1, pedal on the left). The next hole is 6 inches from the first and when the pin is in this position it permits the weights to push the pedal out a distance of 6 inches. There are holes at successive 1-inch intervals permitting a stroke distance up to 13 inches (Fig. 1, pedal on the right). A counter (*L*), activated by the pin, is attached to the apparatus and records the number of strokes. The two upright rods (*M*) permit raising and lowering of the apparatus as desired.

From the Cardio-Pulmonary Laboratory of the National Jewish Hospital at Denver and the Department of Medicine of the University of Colorado School of Medicine.
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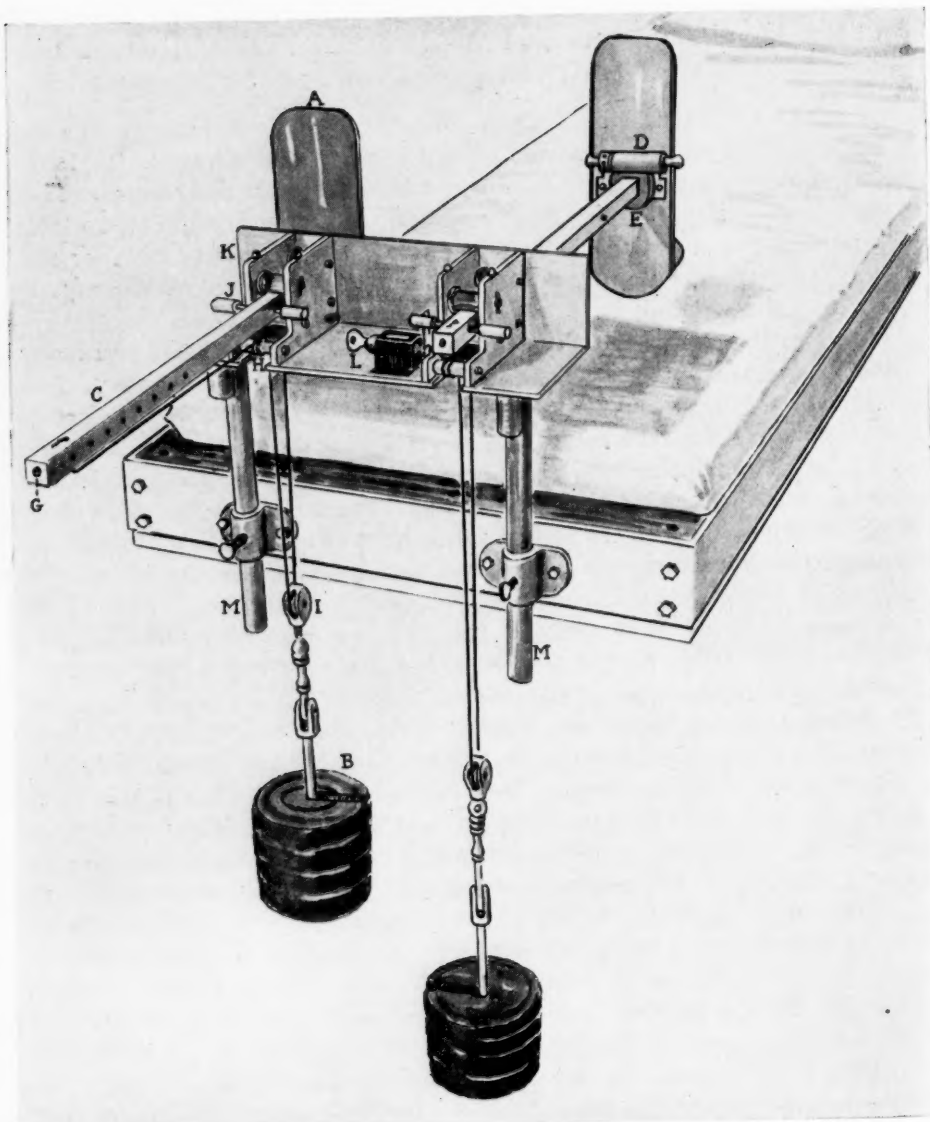

Fig. 1.—Drawing of exercise apparatus bolted in position to the end of a table. See text for explanatory remarks.

In operation it has been found that most patients prefer to push the pedals in together rather than alternately. The stroke distance through which the weights move is adjusted to fit the individual patient. The foot pounds of work

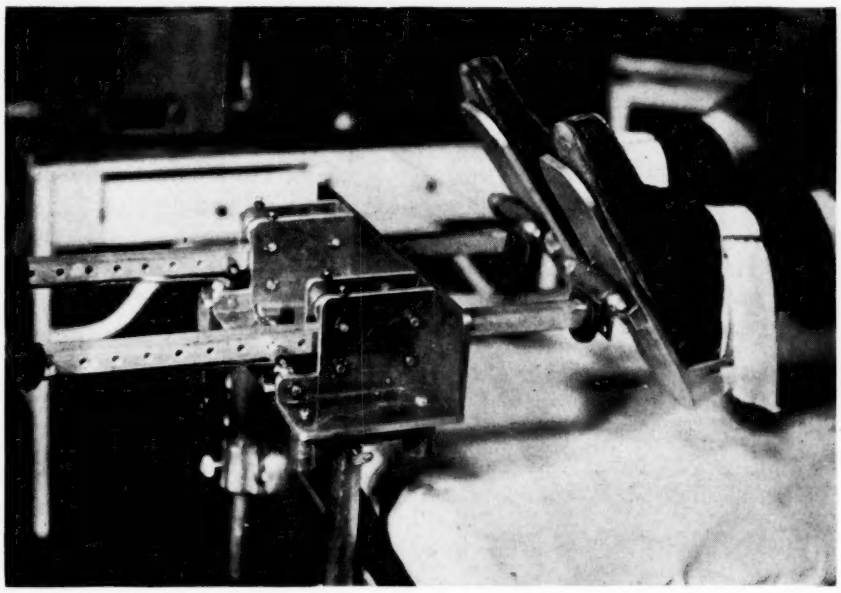

Fig. 2.—Photograph of exerciser in use. Note plantar extension of feet allowed by spring-loaded hinge attached to pedals.

done per minute is easily obtained by multiplying half (due to pulley *I*) the total weight by stroke distance in feet times the number of strokes per minute. As much as 1,150 foot pounds of work per minute can be accomplished on this apparatus.

PYRUVIC ACID DETERMINATION: A MICROMETHOD

MAKEPEACE U. TSAO, PH.D., AND SHIRLEY BROWN, M.S.
ANN ARBOR, MICH.

IN OUR study of premature infants the need for a microscale method to determine pyruvic acid in blood and urine arose. The method by which the most satisfactory results were obtained on a macroscale was that of Friedemann and Haugen[1]; therefore, this was chosen for a microadaptation. Many modifications in technique and apparatus have been introduced to facilitate the actual procedure and to increase the reproducibility of the results. The method to be described requires a sample of 0.1 ml. of either blood or urine; hence, it is possible to use a capillary blood sample for the analysis. The use of capillary blood directly measured into a blood pipette renders possible a quick precipitation of proteins before any change of pyruvic acid content occurs.

METHOD

Apparatus.—

Pipettes. 0.1 ml. Normax, to contain micropipette* for collecting the blood sample.

Constriction type of pipettes† of the following capacities: 18, 33, 75, and 100 cubic millimeters.

Reaction tubes. Pyrex test tubes, 70 by 7 mm., have been tapered at the closed end to facilitate centrifugation and separation of layers. These tubes may be stoppered by No. 000 corks covered with carefully cleaned cellophane or by ground-glass stoppers. In this laboratory the glass stoppers have given more consistent results.

Kahn shaker.

Beckman spectrophotometer and microattachment.‡

Microcuvettes‡ (2 by 10 by 25 mm.).

Reagents.—Reagents used are exactly the same as that described by Friedemann and Haugen[1] except that the sodium hydroxide is approximately 7N. The protein precipitant employed is 10 per cent solution of metaphosphoric acid, and pyruvic acid§ redistilled in vacuo[2] is used to prepare standards.

Collection, Preservation, and Treatment of Sample.—(a) Blood is obtained from a skin puncture of the finger of an adult or the heel of an infant. The skin area is thoroughly cleaned with alcohol and dried, then completely covered with Vaseline (petrolatum U.S.P.). A stab is made with a Bard-Parker Blade #11. From a free-flowing wound a sample is collected directly into a dry and clean 0.1 ml. Normax micropipette. (Triplicate sampling from the same wound is advisable.)

The sample is immediately delivered into 0.5 ml. of protein precipitant in a 5 ml. Pyrex centrifuge tube. The pipette is thoroughly rinsed out with the solution. After thirty minutes of standing, the tubes are centrifuged for ten minutes at 2,500 r.p.m.

(b) Urine samples are collected and preserved according to Friedemann and Haugen.[1] To remove interfering material, 0.75 Gm. of Lloyd's reagent is added to each 10 ml. of

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From the Department of Pediatrics and Communicable Diseases, University of Michigan.

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*Obtainable from Kimble Glass Division, Vineland, N. J., Cat. #37051.

†Constriction pipettes were fabricated in our laboratory but may be obtained from A. H. Thomas Co., Philadelphia, Pa.

‡Microcuvettes and microattachment are obtainable from Pyrocell Manufacturing Co., 207-11 East 84th Street, New York 28, New York.

§Paragon 5300 from Paragon Testing Laboratories, Orange, N. J.

strongly acidified urine; this is mixed thoroughly and centrifuged immediately. As an alternative procedure, especially when only a small urine sample is available, 40 mg. of Lloyd's reagent may be added to the 0.5 ml. of metaphosphoric acid solution into which 0.1 ml. of urine has been pipetted. Excess Lloyd's reagent will interfere with analysis. In case the pyruvic acid content of the urine sample is too high, an adequate dilution should be made to bring it down within the optimum range of this method.

Procedure.—After the centrifugation of the precipitated blood sample or diluted sample of urine, 0.1 ml. of the clear, supernatant metaphosphoric acid solution is transferred to a small reaction tube, using a constriction pipette (see Apparatus). There is sufficient supernatant liquid to run triplicates on each "filtrate."

Thirty-three cubic millimeters of 2,4-dinitrophenylhydrazine reagent are then added to each tube and mixed by shaking. To this mixture is added 100 cu. mm. of xylene. The tubes are stoppered and shaken in a horizontal position for two minutes with a Kahn shaker. A wooden block with holes drilled to accomodate these tubes is used. This block is placed on its side and pressed tightly into place by the adjustable clamp. The stoppered ends of the tubes are pressed against a foam rubber sheet to avoid breakage. Then the tubes are centrifuged at 2,500 r.p.m. for five minutes.

The bottom layer is completely removed and discarded by means of a fine capillary pipette. The hydrazone of pyruvic acid is then extracted from the xylene layer by adding 100 cu. mm. of 10 per cent sodium carbonate solution and shaking for three minutes. Subsequently, the tubes are centrifuged at 2,500 r.p.m. for six minutes.

With a constriction pipette, 75 cu. mm. of the lower carbonate layer is transferred to a microcuvette, 2 by 10 by 25 millimeters. Care should be taken so that no xylene is transferred to the microcuvette or turbidity will result. Then 18 cu.mm. of approximately 7N sodium hydroxide are added and the contents of the cuvette are mixed immediately by blowing air gently through the pipette. The optical density at a wave length of 520 mμ is read in a Beckman spectrophotometer with the microattachment properly aligned. The apparatus is set to 100 per cent transmission (0.000 optical density) by means of a water blank. All readings must be made between two and three minutes after the color is developed.

Cleaning of cuvettes is facilitated by sucking solutions out with a No. 25 hypodermic needle covered with fine elastic tubing* and attached to an aspirator. This technique eliminates the danger of scratching the walls of the cuvettes and the necessity of removing the cuvettes from the carriage. When solutions are sucked out in this manner, it is unnecessary to clean cuvettes between samples unless concentration differences between them are considerable.

Standard solutions containing 1.00, 3.00, and 5.00 mg. per cent pyruvic acid are prepared from the 100 mg. per cent stock standard. These standards and the reagent blank are run simultaneously and in triplicates in exactly the same manner as blood and/or urine samples.

Calculations.—The reagent blank is subtracted from all values, and the cuvette blank, if any, is also corrected to obtain the true optical density corresponding to pyruvic acid in the sample. A linear relationship has been found to exist between the optical density thus obtained and concentration of pyruvic acid standards which lie within the range of 0.5 to 10 mg. per cent.

RESULTS

In all the experiments described here, reaction tubes with glass stoppers were used.

Reagent Blank.—The reagent blank has consistently given high readings, ranging from an optical density of 0.040 to 0.060, or an equivalent of 0.25 to 0.50 mg. per cent.

*This tubing has an inside diameter of .02 in. and an outside diameter of .051 inch. It is obtainable from Surprenant Electric Insulation Company, 84 Purchase Street, Boston, Mass.

Careful study of each reagent revealed that the yellow color of the reagent blank was due to phenylhydrazine reagent, and to this reagent only. An unsuccessful attempt to reduce the color present was made by re-extracting the sodium carbonate layer with xylene; the color persisted.

Extraction.—At first a vigorous agitation by the use of the "whirling nail" device[4] was employed for the extraction of the phenylhydrazone of pyruvic acid from the aqueous layer with xylene. With blood "filtrate," an emulsion invariably formed. Centrifuging at 3,000 r.p.m. for one hour, increasing the density of the aqueous layer by addition of inorganic salt, and adding capryl alcohol, all failed to break up this emulsion completely. Use of the Kahn shaker gives satisfactory extraction without the formation of emulsion, as well as considerable saving of time when large numbers of tubes are to be handled.

A study of the effect of shaking time on the completeness of the two extractions was made. No significant difference in the results was found whether the reaction tubes were shaken at one, two, three, or five minutes.

Color Development and Optimum Range.—Seven normal sodium hydroxide was chosen because it was the lowest normality which would give a deep enough color for a fairly broad range of readings with blood samples and would still be adequate for urine samples of high pyruvic acid concentrations. Thus, the urine and blood samples may be run at the same time and the pyruvic acid content read from the same standard curve. Readings on the final solution were made immediately after the color was developed, five minutes later, and ten minutes later. It was found that there was fading during the ten-minute period. But there was no difference in our final values provided all readings were made after the same time interval following color development. By plotting the light transmission against the logarithm of the concentration according to Ringbom,[3] it was found that this colorimetric method has maximum accuracy between 10 and 90 per cent transmission or what corresponds to approximately 0.5 to 10 mg. per cent pyruvic acid.

Reproducibility of Method.—Repeated analyses were run on each single sample of human blood and urine. The results are shown in Table I.

TABLE I. REPRODUCIBILITY OF MICROMETHOD

BLOOD	PYRUVIC ACID (MG. %)	DEVIATION FROM MEAN	URINE	PYRUVIC ACID (MG. %)	DEVIATION FROM MEAN
A 1	1.83	+0.09	A 1	2.03	+0.05
2	1.76	+0.02	2	2.03	+0.05
B 3	1.70	−0.04	3	1.95	−0.03
4	1.66	−0.08	B 4	1.91	−0.07
5	1.70	−0.04	5	1.99	+0.01
6	1.70	−0.04	6	1.98	+0.00
C 7	1.70	−0.04	C 7	1.89	−0.09
8	1.74	+0.00	8	2.02	+0.04
9	1.74	+0.00	9	1.97	−0.01
10	1.82	+0.08	10	1.99	+0.01
Mean	1.74	±0.04	Mean	1.98	±0.04
Standard deviation		±0.05	Standard deviation		±0.05

The letters A, B, and C refer to different "filtrates" from three separate metaphosphoric acid precipitations of the same sample.

Comparison With Macromethod.—Adult human blood and urine samples were analyzed simultaneously to determine how well the microadaptation checks with the macromethod. Table II lists the results of the comparison.

TABLE II. COMPARISON OF MICROMETHOD WITH MACROMETHOD

SAMPLE	PYRUVIC ACID (MG. %)		SAMPLE	PYRUVIC ACID (MG. %)	
	MACROMETHOD	MICROMETHOD		MACROMETHOD	MICROMETHOD
Blood I	1.90	1.85	Urine I	2.80	3.00
Blood II	1.37	1.48	Urine II	1.00	1.00
Blood III	1.52	1.56	Urine III	0.94	0.99
Blood IV	1.72	1.60	Urine IV	2.47	2.60
Blood V	1.22	1.19	Urine V	1.01	1.11

Values given are averages of triplicate analyses on each sample.

Recovery Study.—Blood and urine samples were precipitated with metaphosphoric acid containing no pyruvic acid and with precipitant containing various but known amounts of pyruvic acid. The "filtrates" were then analyzed in triplicate for pyruvic acid as described under Procedure. The results of this recovery study are shown in Table III.

TABLE III. RECOVERY OF PYRUVIC ACID FROM BLOOD AND URINE

SAMPLE	PYRUVIC ACID IN SAMPLE (MG. %)	AMOUNT OF PYRUVIC ACID ADDED (μG)	AMOUNT RECOVERED (μG)	PER CENT RECOVERY
Blood	1.52	0.50	0.47	94
		1.00	0.94	94
Blood	1.74	1.50	1.53	102
		2.00	2.08	104
Blood	0.93	1.00	1.05	105
		2.00	1.99	100
Urine	2.70	1.25	1.25	100
		2.50	2.55	103
		5.00	4.82	96
Urine	1.78	2.00	1.87	94
		4.00	3.99	100
Urine	1.63	3.00	2.71	90
		6.00	5.87	98

SUMMARY

A microcolorimetric determination of pyruvic acid in blood and urine has been presented. The optimum range and reproducibility of this method were determined. A comparison with the macromethod and a recovery study were made. With this method 0.5 to 10 μg of the acid may be determined.

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A SUCTION TUBE FOR THE COLLECTION OF RESPIRATORY GASES UNDER DIVERSE CONDITIONS

C. A. Forssander, M.D.
Philadelphia, Pa.

THIS modification has been developed as a result of experience with a tube described in an earlier communication,[1] which was later incorporated into an automatic device for the collection of alveolar air by means of a preliminary suction procedure.[2] It presented a means of obtaining samples of alveolar air comparable with Haldane-Priestley expiratory samples without cooperation from the subject, and without interfering with the respiratory pattern.

An advantage of the tube to be described is that, by combining the preliminary suction principle with the smaller evacuation used in the tube referred to previously, it is possible to reduce the time factor by about one third. This is of great importance in all multiple sampling procedures, and in any experiment in which end-expiratory samples are required.

The present apparatus consists (Fig. 1) of a 10 ml. glass barrel into which the sample is taken (the volume of this barrel can be varied according to the size of samples that will be required), with a two-way stopcock of 2 mm. bore at the lower end leading to a mercury reservoir through three feet of plastic tube, containing sufficient mercury to evacuate the sampling barrel. At the upper end of the sampling barrel is a large glass three-way oblique 4 to 5 mm. bore stopcock. The upper bore is reamed out in order to increase the speed of evacuation. Attached to the upper inlet of the stopcock is a rubber bulb (Politzer Air Syringe, No. 288, of 180 ml. capacity). These bulbs have a small inlet ball valve on the side which can be reversed to permit rapid evacuation; it is advisable to apply a piece of rubber tube to the valve, fitted with a spring clip, in order to reinforce the valve if the latter is not efficient by itself.

The rubber bulb is evacuated prior to use by squeezing it with its valve open. The sampling barrel is evacuated by first filling with mercury by lifting the reservoir, closing the upper cock, and then lowering the reservoir until only a small amount of mercury remains in the barrel; the lower cock is then closed.

In order to take a sample the upper stopcock is taken through one complete rotation, which first brings the inlet lead into communication with the rubber bulb and then with the sampling barrel. This not only removes about 150 ml. of gas from the respiratory dead space by means of the automatic opening of the rubber bulb, but also washes out the supply lead with respiratory gas, so that when the rotation of the cock is continued to the point where the barrel is open, an undiluted sample will be taken into the barrel.

In using the tube, it has been found advisable to run the lead to the mouth, and to shut off connection with the room air just before sampling; this latter movement must be carried out rapidly in order not to interfere with respiration.

From the Department of Physiology, School of Medicine, University of Pennsylvania.
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In order to fill both these requirements, a three-way tap was constructed of glass (Fig. 2) with a barrel of 3 in. diameter. The three side tubes are between 1 and 2 in. long and ¾ in. in diameter, one being fitted with a rubber mouthpiece. An opening in the lower side of the mouthpiece tube admits the curved sampling lead, fitted into a rubber stopper. The rotating portion of the tap has an L-shaped passage, so that communication may be made with either side arm, or completely closed off.

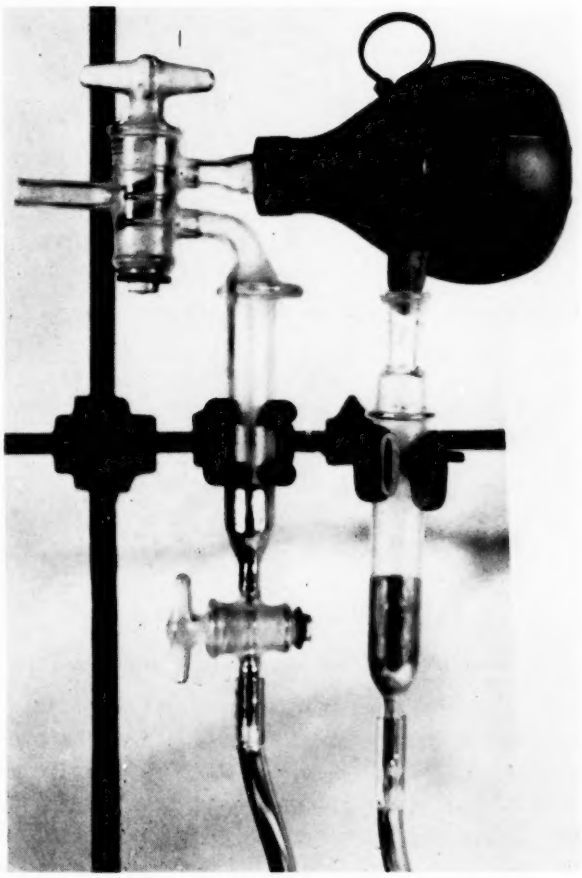

Fig. 1.

The apparatus can be used for collecting respiratory gases at various phases of respiration, and by varying the size of the bulb used, or by using several tubes in parallel, known amounts of gas can be removed from the dead space prior to sampling. By using the second side arm of the tap (Fig. 2) for introducing gas mixtures, the effect of inspired gases on the respiratory gases can be determined. The advantage in this case is that the dead space air can be removed at the end of a normal expiration by means of the bulb, and the subject is then immediately connected with the gas mixture in time for his next inspiration and

does not, therefore, have to inhale the contents of his dead space before coming into contact with the introduced gas. The tube is then used for sampling at the end of any succeeding expiration.

In order that the entire procedure may be carried out with one hand, with a further gain in speed and simplicity, the two-way tap described above may be replaced by the sampling device described in another communication,[2] with the following modifications. The sampling tube described above is attached to the apparatus in the same manner as the earlier one, but the rubber bulb on the

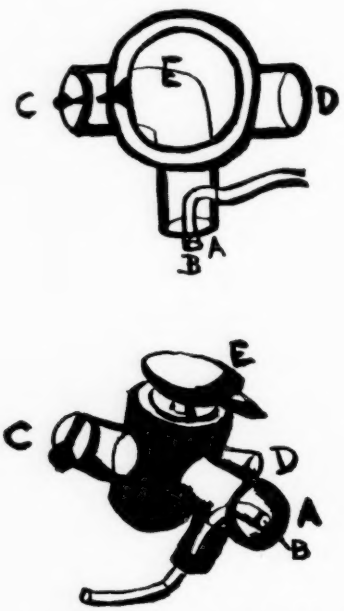

Fig. 2.—*A*, Opening for mouthpiece; *B*, sampling inlet, leading to sampling tube; *C*, opening for breathing room air, with rubber indicator; *D*, opening for gas mixtures; *E*, rotor, with L-shaped internal passage.

apparatus is no longer necessary. Therefore only a single tube need run to the mouth for sampling. At the outlet-inlet a three-way cock or simple slide valve may be fitted which enables the subject to be switched from room air to gas mixture without turning the sampling tap. The cock or slide valve has three openings: one to room air, a small middle one to which is attached a rubber bulb of the same type as is fitted to the sampling tube, and a third to which is attached the supply of gas mixture. The gas mixture may enter from a cylinder, provided that it enters through a rubber bag of about 2 liters capacity fitted with an expiratory valve in order to equalize the pressure.*

For sampling alveolar air whilst breathing room air, the tap of the apparatus is merely turned rapidly through a full rotation at the end of a normal expiration. If the glass cock of the sampling tube has been reamed out efficiently, the movement can be rapid and continuous.

*A suitable piece of apparatus of this type has been constructed by Mr. Otto Hebel, Instrument Maker at the Edward Martin Biological Laboratory, Swarthmore College.

For sampling during the breathing of gas mixtures, the slide valve or cock is pushed from the room air aperture to the mixture aperture at the end of an expiration. This also clears the deadspace in the one movement by means of the rubber bulb. After the required number of breaths of the mixture, the sampling tap is turned through a complete rotation, and the valve returned to room air.

If the Scholander method of gas analysis is used it is advantageous to use a small rubber stopper, perforated by the transfer tube described in an earlier communication,[1] which can be inserted in the inlet of the sampling tube when it is removed from the apparatus for analysis.

This procedure can be carried out without cooperation from the patient and has been found suitable for the determination of venous alveolar air concentrations during the estimation of the cardiac output by a carbon dioxide equilibration method.[3]

The apparatus described was made up in this Department by Mr. J. D. Graham.

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EFFECT OF REFRIGERATION ON THE EHRLICH REACTION IN THE URINE

MARJORIE KNOWLTON, B.S.
BAYREUTH, GERMANY

CONSIDERABLE variation exists in the excretion of urobilinogen in the urine during the twenty-four-hour period of the day, and it has been shown that, when collections are made every two hours, the maximum excretion occurs between two and four o'clock in the afternoon in a significant percentage of normal persons and patients with hepatic disease.[1, 2] Others[3] have reported less constancy in the occurrence of maximum excretion in the afternoon and described greater diurnal variation, not only in groups but also in individual patients. Recent studies in this laboratory of American troops with viral hepatitis corroborated the results of previous investigations[4] of this disease and showed that 53.3 per cent of one group of such patients excreted the maximum amount of urobilinogen in the period between two and four o'clock in the afternoon.

Collection and examination of large numbers of specimens of urine late in the afternoon present certain difficulties in relation to other responsibilities of the wards and laboratory at this time. Because of this, an attempt was made to determine the feasibility of delaying the examination for urinary urobilinogen until the morning following collection of the specimen. It is the purpose of this paper to describe the results of this study.

METHODS AND MATERIALS

One hundred and ninety samples of urine from patients in various phases of viral hepatitis were examined. The Ehrlich reaction was determined according to the method of Watson and co-workers[1] immediately after the collection of urine, in the period from nine to eleven o'clock in the morning. At the time of this test, the remainder of the specimen of urine, which had been collected in a brown bottle, was stoppered and placed in the refrigerator, without light, at a temperature of 37° F. The next morning, after approximately eighteen hours, the urine was allowed to warm to room temperature and the Ehrlich reaction was determined again.

RESULTS

The results of the two tests on 190 specimens of urine were as follows. Before refrigeration, 108 samples of urine had less than 1.4 Ehrlich units (E. U.).* Eighty-two samples had an Ehrlich reaction increased above normal (> 1.4 E. U.), and of these, 69 (84.1 per cent) still had abnormal Ehrlich reactions after eighteen hours of refrigeration. Analysis of the 13 specimens in which the Ehrlich reaction diminished from abnormal to normal amounts after refrigera-

From the Hepatitis Center, 120th Station Hospital, European command.

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*Maximum normal determination in this laboratory.

tion revealed that 11 of them had 1.4 to 2.0 E. U. at the first examination, while only two had more than 2.0 E. U. at the first examination.

Fifty-five samples of urine had more than 2.0 E. U. before refrigeration, and 42 of these had a lower reading in the second test although, as mentioned, only two had less than 1.4 E. U. In 13 of these 42 specimens of urine, the decrease in Ehrlich units in the second test ranged from 20 per cent to 60 per cent; in 29 specimens the decrease was less than 20 per cent.

SUMMARY AND CONCLUSIONS

One hundred and ninety specimens of urine, collected from nine to eleven o'clock in the morning from patients with viral hepatitis, were tested for the Ehrlich reaction immediately after collection, and again after eighteen hours of refrigeration.

Thirteen specimens with Ehrlich units increased above normal at the first test had normal determinations after refrigeration for eighteen hours. Eleven of these 13 specimens had 1.4 to 2.0 E. U. at the time of the first test, and only two of them had more than 2.0 E. U.

The decrease in Ehrlich units after eighteen hours of refrigeration ranged from none to 60 per cent, so that if the original amount in the urine was over 2.0 E. U., the chance of still finding the Ehrlich reaction abnormal (> 1.4 E. U.) was excellent. Since the increase of nonurobilinogen substances which give the color reaction with Ehrlich's reagent is roughly proportional to the increase of urobilinogen, the determination of an abnormal reaction (> 1.4 E. U.) after eighteen hours of refrigeration would suggest that the amount of urobilinogen had been increased above normal.

While not recommended as a standard procedure, the preservation of urine in brown, stoppered bottles overnight by refrigeration for later determination of the amount of Ehrlich units may be permitted, if attention is paid to the limitations outlined here.

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Erratum

In the article by Schneider, Levin, and Haggard, "Carbonic Anhydrase Activity in Sickle Cell Anemia, Sickle Cell Trait, and Pernicious Anemia," in the September issue of the JOURNAL, in Table I, section 4, page 1250, the first figure in the last column should read 1300 instead of 680; the headings of the last columns in Table I should read Carbonic Anhydrase (Enzyme Units/100 c.mm.). In Table III, page 1251, the last column heading should read Carbonic Anhydrase Content of Plasma (Enzyme Units/100 c.mm.).